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ACCENTUATING PATHOGENIC ORGANISMS IN CULTURE BY UTILIZING THE INHIBITORY INFLUENCE OF WHOLE BLOOD.

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CULTURES of bacteria may be prepared to determine what organisms are present in a specimen, and particularly which organisms constitute the ætiological factors in an infection. Yet, with the methods generally employed, both of these purposes may be defeated.

Cultures as ordinarily made fail to differentiate organisms that are pathogenic for the host and hence infecting him, from those that are non-pathogenic for the particular host and of which he is merely a carrier. The organism that predominates in such cultures frequently has little or no pathogenicity for the host, and therefore cannot be regarded as of ætiological significance.

On the other hand, an organism which really is the ætiological factor may fail to grow, even when planted on a suitable culture-medium, through having its growth inhibited by organisms that are non-pathogenic for the patient.

Vaccine prepared from ordinary cultures, therefore, especially when consisting wholly or chiefly of the predominating organisms, may contain little or none of the required antigen, in which case it can have slight therapeutic value. It is doubtless because of this fact that supposedly specific vaccine therapy frequently fails.

The patient's fresh, whole, coagulable blood is believed to possess bactericidal or bacteriostatic power against organisms that are non-pathogenic for him, and to lack it against organisms that are pathogenic for him. Its use, therefore, furnishes a means, other than the guess of the bacteriologist, of separating organisms that are infecting or capable of infecting the host from those of which he is merely a resistant carrier.

When cultures from body recesses, fluids and excretions are made *in vitro* in the patient's whole, coagulable blood, the growth of organisms regarded as pathogenic for the patient harbouring them is promoted and accentuated, while

the growth of those regarded as non-pathogenic for him is inhibited or completely checked.

It has been shown that the whole coagulable blood of certain animals, naturally resistant to infection by a given organism, is bactericidal or bacteriostatic to that organism, while the blood of certain naturally susceptible animals is not.

Heist, Solomon Solis-Cohen and Myer Solis-Cohen (1918) showed that the whole, coagulable bloods of the naturally resistant chicken and pigeon kill the pneumococcus, which, however, grows readily in the whole bloods of the naturally susceptible mouse and rabbit. Bull and Bartual (1920) found the whole blood of the pigeon to inhibit the growth of pneumococci, which property was absent from mouse or rabbit blood; but they found no proof of a true bactericidal action.

Robertson and Sia (1924), using a different method, demonstrated that the blood of naturally resistant animals, at least of the dog and cat, possesses destructive properties for pneumococci, not possessed by the blood of certain naturally susceptible animals.

The whole blood of the rabbit, which is naturally immune to meningococcic infection, was shown by Matsunami and Kolmer (1918) to be bactericidal to the meningococcus, while growth of the organism was vigorous in the blood of the naturally susceptible mouse.

Similar differentiation has been reported by Solomon Solis-Cohen, Heist and Myer Solis-Cohen (1920) between the whole bloods of guinea-pigs and rats, which are naturally susceptible and resistant, respectively, to infection with the diphtheria bacillus.

Bactericidal or bacteriostatic power also develops in the whole blood of animals which are artificially rendered resistant to infection by an organism to which they are naturally susceptible.

Very small doses of killed pneumococci given by Heist and Solomon Solis-Cohen (1919) to rabbits were followed by the rapid appearance of bactericidal properties in their bloods. Believing that the capillary tube (Heist-Lacy) test of Heist and the Solis-Cohens fails to distinguish between bactericidal and bacteriostatic power, Smiley (1923) devised a test-tube method which gave constant and conclusive results in indicating the bactericidal property of the blood of rabbits immunized against pneumococci. By its means he was able to demonstrate bactericidins in the blood of rabbits following the injection of pneumococci. The fact that the titre of bactericidal activity increases in proportion to the amount of pneumococci introduced, suggests, according to Smiley, that there may be some relationship between the bactericidal property of blood and active immunity.

Matsunami (1920) demonstrated the development of bactericidal power against meningococci in the whole blood of mice through progressive inoculations of killed meningococci.

Black, Fowler and Pierce (1920) immunized rabbits against *B. typhosus* and *B. dysenteriæ*, and then seeded their bloods with undiluted virulent cultures of these organisms, incubating the tubes for different periods of time. They found that the organisms were destroyed at the end of five minutes. As the result of comparative studies with various antibodies, they concluded that the

bactericidal power of blood, thus determined, "is the most dependable criterion of the actual immunity of the animal."

Solomon Solis-Cohen, Myer Solis-Cohen and Borow (1921) demonstrated the progressive development of bactericidal power against *B. diphtheriæ* in the blood of rabbits injected with increasing amounts of killed diphtheria bacilli.

BACTERICIDAL OR BACTERIOSTATIC POWER OF WHOLE BLOOD IN MAN.

In order to see whether bacteria known to be commonly harmful to men would grow in human blood more readily than bacteria that are known under ordinary conditions to be practically harmless to men, Heist, Solomon Solis-Cohen and Myer Solis-Cohen (1922) planted in the whole bloods of sixteen healthy men a hæmolytic streptococcus, isolated from a severe leg ulcer, and also planted in the whole bloods of fifteen healthy men a strain of colon bacillus, isolated from fæces. Vigorous growth of streptococci occurred in all but two of the sixteen bloods tested, while almost no growth of colon bacilli occurred in any of the fifteen bloods tested.

These various experiments seem to establish that there is a certain correlation between the ability of bacteria to grow in the fresh, whole, coagulable blood of the species tested and their pathogenicity for that species.

Thinking that a similar correlation may exist between the ability of bacteria to grow in the whole blood of an individual person and their pathogenicity for that individual, I began seven years ago to test with the patient's whole blood all organisms found on culture. Those that grow well in the patient's whole blood I regard as pathogenic for the patient. Those that fail to grow or that grow poorly I regard as non-pathogenic or but slightly pathogenic for the patient.

My clinical results with the therapeutic employment of vaccines made chiefly from the organisms that grow well in the patient's blood seem to bear out this interpretation. Walls (1925), who accurately followed the published technique, also has reported excellent results from the use of vaccine made from the organisms that grew well in the patient's whole blood.

The organisms that are regarded as non-pathogenic for the patient are frequently of pathogenic varieties, but at the moment they probably are not pathogenic for the person harbouring them.

It is, of course, conceivable that should a heavy implantation of organisms non-pathogenic for the patient be made in a relatively small quantity of blood, the amount of bactericidal or bacteriostatic substance present may be insufficient to destroy or inhibit the growth of all of them; and if only a few escape, they may multiply rapidly in the absence of any restraining influence.

THE WHOLE BLOOD TEST FOR MEASURING THE DEGREE OF PATHOGENICITY.

The whole blood test for pathogenicity may be made in one of two ways. In the capillary-tube (Heist-Lacy) method (Heist, Solis-Cohen and Solis-Cohen, 1918), an undiluted culture of each organism tested is run in and out of a capillary tube, and each of five dilutions of the culture—each being one-tenth

of the strength of the preceding one—is similarly run in and out of another capillary tube so that the organisms are left sticking to the walls of the tubes —the first in 100,000 times the concentration of the last. The patient's finger is pricked and the seeded capillary tubes are filled with his blood and sealed. After 24 hours' incubation the tubes are broken, and the blood is stained and examined for the presence of bacteria.

This method has the advantage of yielding approximately quantitative results. Inasmuch, however, as the organisms must first be obtained by ordinary culture, there is always a possibility that the ætiological organisms in an infection may be choked off by other organisms present and may fail to grow up.

The method that I prefer is the test-tube or pathogen-selective method of culture (Solis-Cohen and Heist, 1921). A sterile applicator is applied to the part or substance to be cultured, then stroked over a rich culture medium, such as Loeffler's or serum-agar, and immediately afterward introduced into a small empty, sterile test-tube and rubbed on the bottom and sides of the tube. Blood is then immediately obtained from the patient's vein and about 3 to 5 c.c. are placed in the empty test-tube just inoculated. Both the plain culture and the blood tube are incubated for 24 hours, the former being thereafter placed in an ice-chest to prevent further development. The clotted blood is removed from the blood tube, and with a sterile platinum loop the residual blood at the bottom of the tube is inoculated on one or more tubes of plain culture medium in a thin film on the surface and incubated for 24 hours.

At the end of this period the plain cultures previously placed in the ice-box and those inoculated from the blood tubes are examined for organisms, the amount of growth of each organism being noted.

The two pictures are often quite different. The whole blood, by restraining organisms that are non-pathogenic for the patient, permits a relatively free growth and multiplication of the organisms that are pathogenic for him, even if on the smears from the plain culture they were proportionately few in numbers or even had failed to appear.

When organisms that are not commonly pathogenic to humans predominate in the blood-culture, a second pathogen-selective culture should be taken for verification.

REPORT OF CULTURES.

My records during the past seven years include 863 cultures in which the inhibitory influence of whole blood has been utilized. Most of the cultures were taken by me, the others having been taken by the bacteriologist who studied the cultures. The cultures were studied by Dr. George D. Heist, Dr. Edward Steinfield, Dr. Louis S. Borow, Dr. A. I. Rubenstone and Dr. Fred Boerner. In 20 of the cultures the capillary-tube (Heist-Lacey) method was employed; in the remaining 843 cultures the pathogen-selective (culture-tube) method was used, plates, however, being employed instead of tubes in the ten cultures studied by Dr. Boerner.

In the accompanying chart are given the results of fifteen cultures from the rhinopharynx and tonsils *in situ*, the rhinopharynx and tonsillar spaces after tonsillectomy, the nares, the sputum, the urine and a tooth apex. The

Pathogen-selective cultures of the rhinopharynx and tonsils in situ.

Case.	Medium.	*Streptococcus*	*Staphylococcus*	*M. catarrh.*	Pneumo.	Pseudo-diph.	Leptothrix.	Saccharo-myces	*B. aerogenes.*	*B. coli.*

Pathogen-selective cultures of the rhinopharynx and tonsils in sitû.

Case.	Medium.	*Streptococcus*		*Staphylococcus*		*M. catarrh.*	Pneumo.	Pseudo-diph.	Leptothrix.	Saccharo-myces.	*B. aerogenes.*	*B. coli.*
		non-hem.	*viridans.*	*aureus.*	*albus.*							
1	LBS	...	...	+	...	++	...	+	...	+	...	...
	BT	...	...	+++	...	−	...	−	...	+	..	...
2	LBS	±	...	+	...	++	..	...	...	...	...	...
	BT	−	...	+++	...	+	...	...	...	...	...	...
3	LBS	+	...	...	±	+++	...	+	...	...	...	...
	BT	++	...	...	−	±	...	±	...	...	...	...
Pathogen-selective cultures of rhinopharynx and tonsillar spaces after tonsillectomy.												
4	LBS	−	...	+	...	±	±	...	...	++	...	...
	BT	++	...	±	...	−	−	...	...	−	...	..
5	LBS	...	±	...	+	+	...	±	...	...	...	...
	BT	...	++++	...	−	±	...	−	...	...	...	...
6	LBS	±	...	+	...	+++	±	...	+	...	...	...
	BT	−	...	+++	...	−	−	...	+	...	...	...
Pathogen-selective cultures of sputum.												
7	LBS	+	++	...	+	...	...	...	...	...	...	...
	BT	−	++	...	+	...	...	...	...	...	...	...
8	LBS	+	...	...	...	+	...	+	+	...	...	...
	BT	++	...	...	...	−	...	−	−	...	...	...
9	LBS	+	...	+	...	+	+	...	...	...	...	...
	BT	++	...	±	...	±	±	..	...	...	...	...
Pathogen-selective cultures of urine.												
10	LBS	...	...	−	...	...	...	...	...	...	+	...
	BT	...	...	+	...	...	...	...	...	...	−	...
11	LBS	...	...	+	...	...	...	...	...	...	...	++
	BT	...	...	+	...	...	...	...	...	...	...	±
Pathogen-selective cultures of tooth.												
12	LBS	+	...	+	...	...	...	...	...	...	...	...
	BT	−	...	++	...	...	...	...	...	...	...	...
Pathogen-selective cultures of nares.												
13	LBS	...	...	+	...	+	...	...	...	...	...	...
	BT	...	...	++	...	±	...	...	...	...	...	...
14	LBS	...	...	±	±	...	...	...	...	...	...	...
	BT	...	...	+	−	...	...	...	...	...	...	...
15	LBS	...	...	+	...	...	±	...	...	...	...	...
	BT	...	...	++	...	...	−	...	...	...	...	...

EXPLANATION OF TABLE.

Media.—BT, whole blood test-tube test (pathogen-selective method); LBS, Loeffler's blood-serum.
Recording organisms.—±, few or very few; +, moderate amount; + +, large numbers; + + +, + + + +, very large numbers; —, absent.

approximate quantity of each organism present is indicated, so that one can see at a glance whether in the blood-culture its growth has been retarded or exaggerated.

None of the persons cultured were healthy or normal.

In many of the cultures it will be noted that the organism predominating on the blood-culture is not the one that predominates on the plain culture.

Several of the cultures show that an organism may grow up on the blood-culture that was not observed on the plain culture.

CONCLUSIONS.

1. Cultures as ordinarily made fail to differentiate organisms that are pathogenic for the host, and hence infecting him, from those that are non-pathogenic for him, and of which he is merely a carrier.

2. Ordinary cultures may even fail to contain the ætiologic factor in an infection.

3. In the pathogen-selective culture the patient's whole coagulable blood is utilized to inhibit the growth of organisms that are non-pathogenic for him, thus permitting the free growth of organisms that are pathogenic for him.

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THE EFFECT OF EXTRACTS OF THE ANTERIOR LOBE OF THE PITUITARY GLAND ON THE LIVER.

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DURING certain observations on the effects of pituitrin on fat metabolism (Coope and Chamberlain, 1925), rabbits' livers were examined histologically and chemically for fat, and a large excess was found in most cases. Experiments were then made to determine whether this fatty infiltration was specifically due to pituitrin, or whether it could be produced by other ductless gland extracts.

Amongst the latter, extracts of the anterior lobe of the pituitary were used, generally that extract prepared by Messrs. Parke Davis called "antuitrin." Whilst no chemical or histological evidence of an increased fat content in the liver was furnished by these experiments, certain other peculiar histological changes were noted, and further work was undertaken to determine the nature of these changes, and the conditions under which they occur.

The results obtained are described in this paper.

TECHNIQUE OF EXPERIMENTS.

The majority of the experiments were made on rabbits, and all descriptions refer to these animals unless otherwise stated. A few experiments were made with guinea-pigs and frogs.

The animals were fed for several weeks before use on a standard diet of bran, green-stuff and water until death. In most cases male animals were preferred so as to avoid any possible complicating influences of pregnancy or lactation.

"Antuitrin" was used in most of the experiments, and was kindly supplied by Messrs. Parke Davis and Co., though occasionally other anterior lobe pituitary extracts were employed.

The extract was given subcutaneously mixed with a solution of gum arabic, as this method of administration was found in previous work to prevent any ill-effects which might ensue from too rapid an absorption of the extracts, without in any way interfering with the results of the experiments.

The animals were killed by a blow on the neck at intervals of 5 to 19 hours after the injection, and the liver was rapidly excised to prevent glycogenolysis.

Portions of the liver were taken for chemical analysis, estimations being made in various experiments of glycogen, water, and in some cases of cholesterol and phosphorus.

Other portions were preserved in fixatives, formalin, Carnoy's reagent or absolute alcohol according to the nature of the histological preparations to be made. Paraffin sections were made and stained with Heidenhain's stain or hæmatoxylin and eosin for general examination. Colloidin sections were prepared for glycogen staining, and frozen sections for fat. Glycogen was stained by Best's carmine and the iodine methods, fat with Scharlach R. The chemical examinations were undertaken in an endeavour to correlate the histological with the chemical changes. Glycogen was estimated by Pflüger's (1904) method, water by a method to be described later, cholesterol by Grigaut's (1911) method, and phosphorus by a modification of the Bell-Doisy (1920) method.

Fourteen control experiments were made (*a*) on five perfectly normal animals without any injection, (*b*) on three animals injected with the solvent used in the preparation of "antuitrin," and (*c*) on three animals injected with 1% acetic acid solution, as this acid is contained in the "antuitrin" solvent. The solutions injected were in all cases mixed with gum-arabic solution. The control animals and the injected animals were housed under identical conditions, and were fed on a similar diet, except in the case of three animals which were fed on a diet of carrots to increase the glycogen-content of the liver.

HISTOLOGICAL CHANGES.

Fifteen experiments were made with "antuitrin," and the anterior lobe pituitary extract (A.L.P.E.) change was found in eight of them. Of the other seven cases four livers were perfectly normal in structure, but three showed some indistinctness in the outline of the cells, and the nuclei stained badly.

These changes suggest a mildly degenerative process, and were sometimes seen in those animals injected with the solvent of "antuitrin" and in those injected with acetic acid solution, and may therefore be due to the acetic acid present in "antuitrin." They are, however, quite different from the group of changes now to be described and called for convenience the "A.L.P.E." change.

The "A.L.P.E." change can best be appreciated from the accompanying microphotographs. The individual cells of the liver appear swollen, and when measured their mean diameter was found larger than that of normal liver-cells in the approximate ratio of 11 : 8. The cells were rounded in shape instead of polygonal, and the cytoplasm stained less intensely than normal and contained only a few granules. Instead of the usual disposition of the liver-cells in columns radiating from a central vein, the cells had lost their columnar arrangement and were grouped together indiscriminately, apparently due to an obliteration of the intercolumnar spaces by the swelling of the cells.

In places, also, the central veins seem to be obliterated.

The nature and significance of this group of changes is discussed later.

CHEMICAL CHANGES.

The "A.L.P.E." change resembled quite closely those changes associated with a glycogenic infiltration of the liver—a condition which, as Turnbull (1920) has pointed out, may easily be mistaken for certain degenerative lesions of the liver. Table I, however, will show that there is no excess of glycogen in the "A.L.P.E." livers. Moreover, no excess was demonstrable histologically.

Estimations of cholesterol were made in several cases owing to the presence of doubly refractile granules noticed in two experiments where the sections were examined by polarized light. No excess or decrease of cholesterol was found. Phosphorus was also estimated in some experiments, as a rough indication of any alteration of the lecithin-content of the liver. Again no excess or decrease was found.

In the case of water estimations, however, it will be observed from Table II that the water-content of the "A.L.P.E." livers was appreciably higher than

that of the control livers, averaging 75·1 % in the former and 70·6 % in the latter.

TABLE I.—*Glycogen Content of Livers.*

Control animals.		Animals injected with "antuitrin."	
Rabbit No.	Glycogen %.	Rabbit No.	Glycogen %.
3	5·66	6	6·36
4	9·66	7	8·67
5	5·10	12	4·22
8	5·19	19	2·46
11	5·71	20	4·58
...	...	28	5·25
Average	6·26	Average	5·26

TABLE II.—*Water Estimations.*

Control animals.		"Antuitrin" animals.	
Rabbit No.	Water %.	Rabbit No.	Water %.
18	72·8	19	73·3
21	68·4	20	81·0
...	...	25	71·2
Average	70·6	Average	75·1

TABLE III.—*Phosphorus Estimations.*

Controls.		"Antuitrin" animals.	
Rabbit No.	Phosphorus mgm. %.	Rabbit No.	Phosphorus mgm. %.
11	303	12	294
16	228	13	370
17	332	14	303
18	290	15	289
21	275	19	329
...	...	20	306
...	...	25	208
Average	285	Average	295

TABLE IV.—*Cholesterol Estimations.*

Controls.		"Antuitrin" animals.	
Rabbit No.	Cholesterol mgm. %.	Rabbit No.	Cholesterol mgm. %.
29	238	20	283
30	139	25	312
33	225	26	226
35	354	28	361
Average	239	Average	295

A further series of 10 normal animals gave liver cholesterol as varying between 183 to 355 mgm. %.

The water was estimated by mincing the liver and smearing in a very thin layer over a flat piece of glass, and drying at a temperature of approximately 120° C. until it was of constant weight.

In the experiments recorded in Table II the results of this method, though few, were very reliable. It would be expected that this increase of water-content would lead to a relative diminution of the solid constituents, but owing to the large variation of these constituents in normal livers, it is not possible to attach any significance to such small variations in the content of glycogen, cholesterol or phosphorus as were present. No gross excess or decrease, however, was present.

Changes in other organs.—Histological preparations were also made of the kidneys, myocardium and suprarenal bodies of several animals injected with "antuitrin," but no histological changes were found.

Experiments on other animals.—In one of the two cases where guinea-pigs were used the liver showed an "A.L.P.E." change, closely resembling that seen in rabbits. In frogs, as would be expected from the simple structure of the liver, no such changes were observed.

ATTEMPTS TO PRODUCE THE "ANTUITRIN" CHANGE BY OTHER MEANS.

1. *By other ductless gland extracts.*—The "A.L.P.E." change was not produced by injections of pituitrin (posterior lobe pituitary secretion), nor by insulin given to the point of producing convulsions, nor by feeding with thyroid gland for 2–7 days. No comparable changes whatsoever were produced.

2. *By bacillary injections.*—Prof. J. M. Beattie suggested that changes similar to the "A.L.P.E." change might be produced by injection of *B. sporogenes*. With the object of comparing the two effects rabbits were injected intravenously with a culture of *B. sporogenes* and were killed at periods of 2–8 hours after the injection. Sections of the liver were made immediately after death, and also at intervals of ½ hour up to 6 hours later. The sections were stained with hæmatoxylin and eosin and with Gram's stain.

In no case were any changes noted in the sections taken at death, so it is presumed that no changes are produced in the liver during life by the organisms. In the post-mortem specimens various degrees of disintegration of the liver were found, from slight swelling of the cells and separation at the intercellular spaces, to complete destruction of the structure of the organ with the presence of bacteria. In no case, however, was there any change comparable with the "A.L.P.E." change.

Sections of a sheep's liver dead of dysentery also failed to show any indication of the "A.L.P.E." change.

3. *By injection of bile-salts.*—It has been shown by Davidman and Dolley (1921), and others, that rats injected with a 5% solution of bile-salts develop a type of cloudy swelling which will be referred to in the discussion, and it was thought that similar changes might be produced in rabbits, and would afford an interesting comparison with the "A.L.P.E." change.

The first rabbit received 3 c.c. of 5% bile-salt solution subcutaneously on two consecutive days. At the end of 24 hours it became ill, and died after 48 hours.

The liver showed the pale "washed-out" appearance characteristic of cloudy swelling. Histologically, the liver-cells showed increased granularity, indistinctness of outline, and degenerative changes in the nucleus—changes quite unlike those produced by "antuitrin."

The second animal was injected with 1 c.c. of 5% bile-salt solution daily for 6 days. It showed malaise, but was not severely ill.

It was killed on the seventh day. The liver was slightly paler than normal, and showed histological changes like those in the first rabbit, though much less intense.

The third animal had a single dose of 4 c.c. 5% bile-salt solution, and was killed 17½ hours later. There was little, if any, malaise before death. Histologically the liver was normal. From these experiments it will be seen that whilst bile-salt injections produce cloudy swelling of the liver in rabbits, it is probably different in type from that produced in rats, and does not resemble the "A.L.P.E." change.

The outcome of these experiments is that so far it has not been possible to reproduce the "A.L.P.E." change by other means than by the injection of anterior lobe pituitary extracts.

DISCUSSION.

The nature of the "A.L.P.E." change is still somewhat uncertain, but the writer regards it as a somewhat unusual type of cloudy swelling—a type which bears a close relationship to the condition described by some authors as "dropsical degeneration."

This view is based on the œdematous appearance of the cells and the increased water content of the livers.

It has already been mentioned that a similar histological picture is presented by glycogenic infiltration of the liver in rabbits, but the possibility of the change being due to glycogen has been excluded, and a search through the literature has discovered no changes other than cloudy swelling which at all resemble it.

Cloudy swelling is still regarded by many authorities as essentially a degenerative process, but there is considerable evidence that in its early stages it is merely an expression of over-activity on the part of some tissue which has been stimulated unduly—that it represents in fact a protective response on the part of the animal. Adami (1909) was one of the first to emphasize this conception, and regarded cloudy swelling as a degenerative change only in its later stages where the abnormal stimulus had been extreme.

A well-known example of cloudy swelling produced by an abnormal but physiological stimulus is furnished by the experiment of Favre in 1892, and worked out in more detail by Schilling (1894), in which ligature of the left renal vein in animals was followed by cloudy swelling of the opposite kidney, produced presumably by the increased amount of work put on one kidney by interference with the function of the other. Albrecht (1903) obtained similar results by ligature of the ureter on one side.

In a valuable contribution to the study of cloudy swelling, Davidman and Dolley (1921) have developed these views further. They point out that cloudy swelling is a condition produced in many diverse ways, and characterized by two stages—firstly a stage of excitation, and later a stage of depression.

Either of these stages may be produced alone, or the excitation stage may pass insensibly into the depressive stage.

The stage of excitation is characterized by swelling of the cells due to accumulation of water. Adami regarded this imbibition of water as an essential part of cloudy swelling, and attributed it to the dissociation of colloids in the cytoplasm, producing crystalloids which led to osmotic diffusion of water into the cells.

From the description of the "A.L.P.E." change it will be seen that it corresponds quite closely with the excitation stage of cloudy swelling.

The stage of depression is marked by diminution in the size of the cells, with turbidity and accumulation of albuminous granules in the cytoplasm.

This depressive stage is quite unlike the "A.L.P.E." change, though, as previously mentioned, some minor changes of the same nature were occasionally found after the injection of "antuitrin," and especially after the injection of "antuitrin solvent" or dilute acetic acid. They were also found in the bile-salts experiments.

Both stages of cloudy swelling may be produced by the same stimulus, but if the stimulus is mild or acts for a short period of time it tends to produce only the excitation stage, whilst if it is a powerful stimulus or one acting over a long period of time it will probably produce the depressive stage.

Certain stimuli may, however, irrespective of their dosage or the frequency with which they are applied, produce either the excitatory or the depressive stage alone: for example, subcutaneous injection of bile-salts into rats usually results in cloudy swelling of the excitatory type.

This excitatory stage having been regarded by some writers as an expression of increased physiological function, bile-salts have been used therapeutically to stimulate liver function. By analogy it would seem that anterior lobe pituitary extracts, in producing the excitatory stage of cloudy swelling may also act as a stimulant of liver function, and possibly might be of value therapeutically.

Here it may be observed that animals injected with "antuitrin" showed no apparent deviation from normal health—a fact supporting the view that the "A.L.P.E." change is physiological rather than pathological.

Anterior lobe pituitary extracts, however, differ from other stimuli producing similar effects, in that the changes induced by it were confined to the liver and did not occur in other organs. Cloudy swelling produced by other stimuli such as bile-salts, excessive temperature, etc., usually affect several organs.

The anterior lobe of the pituitary gland is regarded on clinical grounds as playing an important part in the metabolism of growth, both skeletal and sexual. Experimental work by Brailsford Robertson (1916) with a substance prepared from the anterior lobe which he terms "tethelin" accords with these views.

It is interesting to note that recent work by Kestner and Liebeschutz

(1926) indicate that anterior lobe pituitary extracts exert a specific dynamic action.

The production of the "A.L.P.E." change therefore lends further support to the supposition that anterior lobe pituitary extracts are physiologically active.

It is possible that the acetic acid present in the extracts has helped in the production of the "A.L.P.E." change, though hardly to any marked degree, as the change was never appreciable in animals injected with acetic acid alone or with the "antuitrin" solvent, kindly supplied by Parke Davis & Co., which contains acetic acid.

Probably therefore the change was due to some specific constituent of the anterior lobe of the pituitary which was present in the extract.

On the other hand, the occasional presence of what appeared to be the depressive stage of cloudy swelling in some animals injected with "antuitrin" may have been due to the acetic acid present in the extracts, for injection of acetic acid or "antuitrin" solvent frequently resulted in this change.

Finally, in those animals injected with "antuitrin," in which no changes occurred in the liver, one must suppose either that the individual animals vary in their response to the extract, or that the active substance in the extract which produces the "A.L.P.E." change varies in potency.

SUMMARY OF CONCLUSIONS.

1. Injection of anterior lobe pituitary extracts gives rise to a group of striking histological changes in the liver, called for brevity the "A.L.P.E." change.

2. The evidence available suggests that this "A.L.P.E." change is dependent on the presence in the extract of some special active substance derived from the pituitary gland.

3. Chemical and histological investigations indicated that the variations of the "A.L.P.E." livers in total fatty acids, glycogen, total phosphorus and cholesterol hardly exceed those of normal rabbits' liver, but there is a significant increase in the water-content.

4. The similarity between the "A.L.P.E." change and the excitatory stage of cloudy swelling is pointed out, and it is argued from this similarity that the "A.L.P.E." change is a manifestation of increased functional activity of the liver, and that anterior lobe pituitary extracts may possibly be of value therapeutically.

5. Nothing resembling the "A.L.P.E." change has been obtained in the myocardium or the kidneys.

6. Nothing similar to it could be obtained in rabbits by injection of extracts of the posterior lobe of the pituitary, or of insulin, or of bile-salts, nor by thyroid feeding, nor by injection with *B. sporogenes*.

I wish to acknowledge here much valuable help and advice from Prof. W. Ramsden, in whose department this work has been carried out.

Prof. Glynn, Prof. J. M. Beattie and Prof. C. E. Walker have also given me valuable assistance, and my thanks are due to them.

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DESCRIPTION OF PLATE.

FIG. 1.—Normal liver, × 50.
FIG. 2.—"A.L.P.E." change, × 50. Showing general configuration of liver-lobules.
FIG. 3.—Normal liver, × 600. Shows the characteristic columnar arrangement of the liver-cells.
FIG. 4.—"A.L.P.E." change, × 600. Shows loss of cell columns owing to œdematous swelling of the cells. Note also the empty appearance of the cells owing to fewer and more faintly staining granules.

COCCAL FORMS OF THE DIPHTHERIA BACILLUS.

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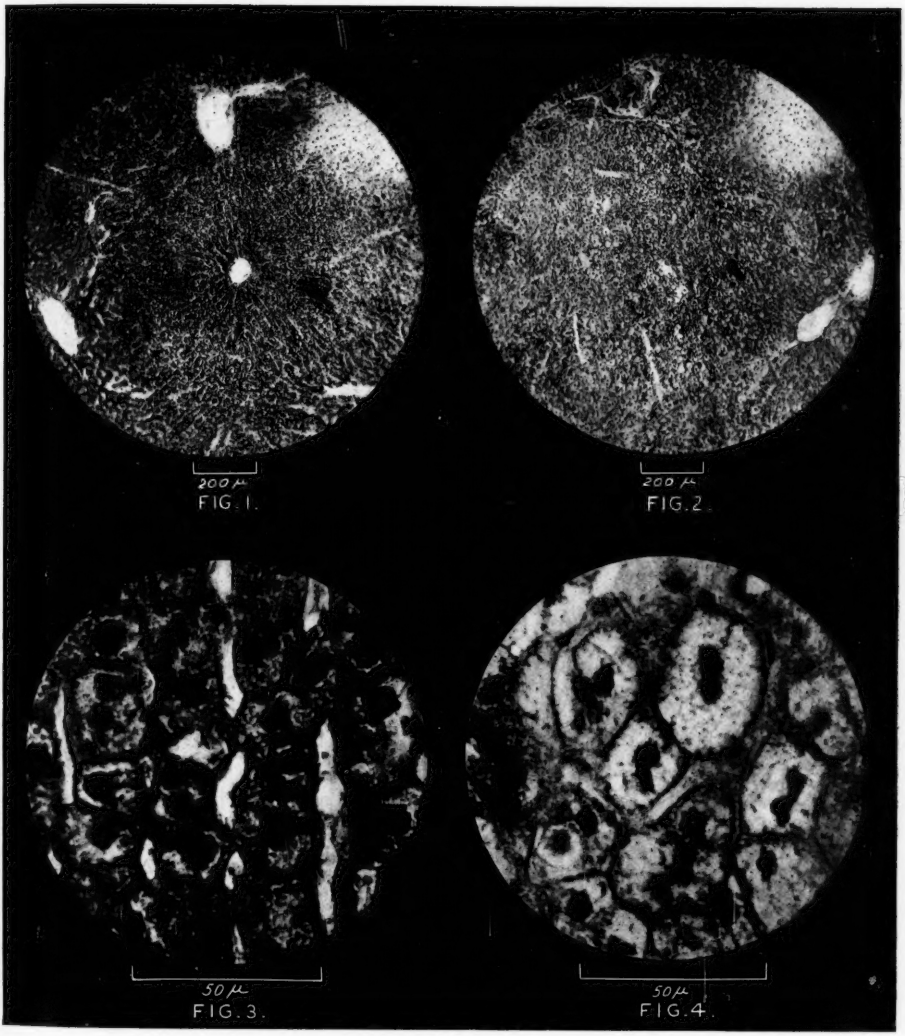
Received for publication March 30th, 1927.

FOR several years diphtheria toxin, prepared from the "Park and Williams No. 8" strain of *C. diphtheriæ*, has been used for the immunization of horses at these laboratories. From time to time smears of broth cultures of this organism used for toxin production have contained coccoid forms resembling micrococci, although irregularity in outline or staining, or the presence of intermediate forms between bacilli and cocci, has usually suggested a bacillary origin. These cultures of doubtful purity, when plated on Loeffler's blood-serum, have shown only characteristic dome-shaped colonies consisting entirely of bacilli.

Coccoid forms of diphtheroids have been described by Mellon (1917). Heinemann (1917) noticed the replacement of typical bacillary forms by coccus forms in certain of his cultures on beef, veal or horse-meat broth. His coccus-like growths produced similar toxin to his bacillary forms but of relatively low potency. On Loefflers's blood-serum and veal glucose agar


200 μ
FIG. 1.
200 μ
FIG. 2.
50 μ
FIG. 3.
50 μ
FIG. 4.

plates bacilli were present; on veal glucose agar slants they were invariably replaced by a certain number of coccal forms.

Crowell (1926) has studied the variants from a single virulent diphtheria bacillus, and has described coccoid forms, both virulent and avirulent, on Loefflers' blood-serum slants amongst the descendants of the original organism. He concludes that a virulent organism under favourable conditions always throws virulent offspring, but under unfavourable conditions virulent and avirulent, and that "morphological types of the diphtheria bacillus have no hereditary significance, and have no relation to virulence."

Yarisawa (1926) has described coccoid formation in 14 % of his collection of strains isolated from diphtheria patients. This was manifested only on certain media, especially in fresh blood-agar cultures transplanted from blood-serum media. When a suspension of coccal forms was injected into experimental animals there was definite evidence of toxicity, and bacillary forms were recovered post-mortem.

Yarisawa believes that insufficient heating of serum media is an important factor in inducing the coccal change, and was able to produce coccal forms of some of his strains by using insufficiently-heated Loeffler's medium. In ordinary bouillon he observed bacillary forms only.

The broth used for toxin-production at these laboratories has been Douglas's tryptic digest, modified by Hartley, and subsequently by Watson and Wallace. Digestion has usually been allowed to take place at 37°–40° C. for 3½ hours, but recently a three days' digest of horse-muscle at room temperature has been tried with favourable results. In the latter medium coccal forms are rather more frequent, but even then are rarely seen. A culture was obtained, however, some months ago consisting almost entirely of them. Through the kindness of my colleagues, Dr. A. F. Watson and Miss E. D. F. Langstaff, I have been enabled to study this culture in detail.

The culture consisted of a five days' growth of the organisms at 34° C. on a three days' tryptic digest of horse-muscle at room temperature. The medium had been sterilized by filtering through a Seitz press and autoclaving for twenty minutes at a pressure of 11 lb.

CHANGES OF MORPHOLOGY ON DIFFERENT MEDIA.

The original broth culture consisted for the most part of minute bodies arranged in clumps (Fig. 1). Many also were in diplococcal or streptococcal formation as if arranged along the bodies of bacilli, although the intermediate protoplasm was not seen. They only feebly resisted decolorization by Gram's method, and did not show metachromatic staining with Neisser's stain. A few slender and indistinct rod-shaped organisms were also present.

Subcultures were made from the original broth culture on to tubes of various media, from which plates were also prepared at frequent intervals to establish the continued purity of the strain. The main implantations were from (1) Loeffler's medium to Loeffler's medium, and (2) agar, prepared with trypsin digest broth as basis, to agar, with subcultures from time to time on to trypsin digest broth, serum digest broth, glucose digest broth, steamed Witte peptone broth, Witte peptone agar, and blood-agar. The Loeffler's medium was prepared by the addition of one part of sterile glucose digest

broth to three parts of normal horse-serum, the mixture being inspissated at 80° C. for one hour on two consecutive days.

On Loeffler slants and plates the sudden disappearance of the coccoid forms was most striking. The first generation consisted of Neisser-negative bacilli with some degree of beading; the second and subsequent generations showed typical Neisser-positive bacilli (Fig. 2).

On nutrient agar, prepared with tryptic digest broth as basis, coccal forms persisted for several generations (Figs. 3 and 4). They became steadily less numerous, until by the sixth successive subculture they had disappeared. None of the bacillary forms on "digest agar" had metachromatic bodies which could be stained by Neisser's method. It was found, however, that by subculturing from agar on to Loeffler's medium typical Neisser-positive organisms appeared within 24 hours. Subcultures of bacillary forms into digest broth led to the reappearance of the coccoid formation in the first generation (Fig. 5), the first coccoid forms being seen about half-an-hour after inoculation.

In the first transplant from the original broth on to agar, prepared with Witte peptone broth as basis, beaded bacilli with some degree of Neisser-positive staining displaced all the coccal forms (Fig. 6). In Witte peptone broth alone, however, two successive subcultures from the original broth contained numerous coccal forms. They also persisted in Hiss serum water, Witte peptone water, serum broth, glucose broth and blood-agar, suggesting that once an organism has assumed the coccal form, it tends to retain that formation unless it is placed on an eminently favourable medium such as Loeffler's blood-serum.

The colonies on plates of Loeffler's blood-serum were always bacillary. On plates of other media, however, colonies have been observed consisting of mixtures of coccal and bacillary forms. One batch of "digest agar" plates was of special interest. Two types of colonies appeared, one of which was much larger than the other; microscopically, one of these types was entirely bacillary, the other, the smaller variety, a mixture of coccal and bacillary forms.

A few freshly isolated strains of virulent and avirulent diphtheria bacilli and Hofmann's bacillus were subcultured on to the original broth (3 days' digest at room temperature), and assumed coccoid formation within 24 hours. Like the "Park and Williams No. 8" strain, they reverted almost at once to their typical morphology on a further transplantation to Loeffler's medium.

An attempt was made to induce coccoid formation direct from Loeffler slants by the use of media other than trypsin digest broth. As recommended by Yarisawa (1926), Loeffler's medium, which had been over-heated or insufficiently heated, and fresh blood-media, especially guinea-pig blood-agar, were inoculated with certain virulent and avirulent strains. It was found that by alterations in the medium, variations in morphology could readily be induced, but in no instance was there the extreme morphological change observed in the original batch of digest broth.

It should be stressed at this point that on Loeffler's medium, in our experience, even when a few cocco-bacillary or coccoid forms were present, the

remainder of the organisms were so nearly typical that the identification of the organism as *C. diphtheriæ* was never in doubt.

Miss Langstaff has occasionally observed marked coccal formation in one bottle of a toxin batch, inoculated at the same time and under the same conditions as many others in which only bacillary forms were present. In an attempt to explain this variation, experiments were carried out with negative results with dirty bottles and bottles containing fat or soap; nor did an increase in the phosphate or chloride content of the broth nor the addition of fruit or vegetable juice induce the coccal change.

VIRULENCE AND TOXIN PRODUCTION OF COCCAL FORMS.

Coccal forms of "Park and Williams No. 8" and two other strains which had been virulent when first isolated in the bacillary form were re-tested for virulence. Washed suspensions of the growths in digest broth were found to be fully virulent by the subcutaneous and intracutaneous methods of testing. In the subcutaneous test, organisms recovered from the site of injection were mainly bacillary in shape, indicating a tendency to reversion in the living body.

Forty-eight colonies from Loeffler plates, which had been inoculated from broth-cultures of three strains showing coccal forms, were also tested for virulence by the intracutaneous method. All were fully virulent, there being no evidence of any variation from colony to colony. These observations were the outcome of Crowell's hypothesis that a virulent organism under favourable conditions always throws virulent offspring, and under unfavourable conditions virulent and avirulent; once avirulent they do not again become virulent. If coccal formation is a response to an unfavourable environment, on this hypothesis one would have expected some of the colonies on the Loeffler plates to be avirulent, or at all events of intermediate virulence. No variation in virulence was, however, observed.

On several occasions good toxin has been obtained from cultures containing coccal forms. Whenever a bottle containing coccal forms has not produced as highly toxic a filtrate as other bottles of the same batch containing bacillary forms only, the growth in that bottle has been less vigorous—that is, fewer organisms were present. We do not know, therefore, if coccal forms individually are as good toxin-producers as bacillary forms.

SUMMARY.

(1) Coccal forms have appeared from time to time in tryptic digest-broth cultures of *C. diphtheriæ*.

(2) A culture of the "Park and Williams No. 8" strain on a 3 days' tryptic digest of horse-muscle at room temperature consisted almost entirely of coccal forms. This was a unique instance of extreme coccoid formation amongst several thousand broth-cultures examined. A large number of coccal forms were also produced when recently isolated virulent and avirulent strains were sown in this broth.

(3) The coccal forms disappeared on the first subculture on Loeffler's medium and on agar prepared with Witte peptone broth as basis. They were still

present in the fifth generation on agar prepared with digest-broth, but had disappeared in the sixth.

(4) Typical bacillary forms were most readily induced on Loeffler. Although coccal forms have been observed on this medium when it has been insufficiently heated, the remainder of the organisms have been so nearly typical that the identification of the cultures as *C. diphtheriæ* was never difficult.

(5) Coccal forms are virulent and toxigenic. There is no evidence that they are indicative of racial degeneration of the organisms.

I wish to thank Dr. A. F. Watson and Miss E. D. F. Langstaff for the original culture, estimations of toxicity of filtrates, and much helpful criticism. I am indebted to Dr. A. C. Stevenson of the Wellcome Bureau of Scientific Research for the micro-photographs.

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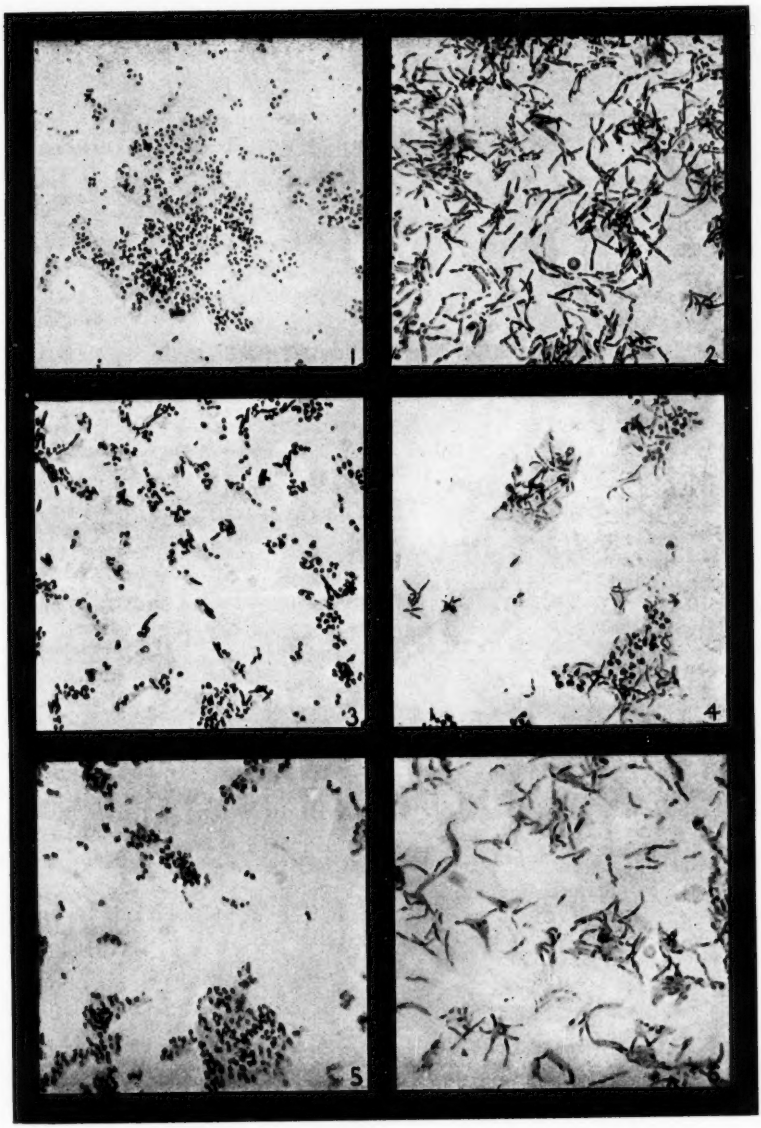
DESCRIPTION OF PLATE.

Fig. 1.—Tryptic digest broth culture. Gram stain.
Fig. 2.—4th subculture on Loeffler's medium. Methylene blue stain.
Fig. 3.—1st subculture on agar (tryptic digest broth basis). Neisser's stain.
Fig. 4.—3rd subculture as in Fig. 3.
Fig. 5.—Subculture from Loeffler's medium to broth as in Fig. 1.
Fig. 6.—1st subculture on agar (Witte peptone broth basis). Gram stain.



Parish.

UNIV. OF MICH.

PRESERVATION OF THE VIRUS OF HERPES.

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THE method which is commonly used for the preservation of the virus of herpes consists in glycerination of virulent brain and its storage in a cold room or in an ice-chest. It is the practice of most workers to dilute the glycerin with normal saline, the figures ranging from 20 to 60 per cent., but pure glycerin is also occasionally used.

In the course of an experience of six years with this virus it used to be the practice of the author to place portions of virulent brain in small, wide-necked, glass-stoppered bottles containing a volume of 50 per cent. glycerin in saline equal to five to six times at least that of the brain. It was found that under these conditions in an ice-chest whose mean temperature was + 9°C., the diluted glycerin became very rapidly acid, and that the brain became non-infective within a couple of months. The diluted glycerin was next brought to a pH of 7·4 with NaOH and the liquid in the bottle was changed once a week. Under these latter conditions the time that the brain remained infective was increased to three to four months at + 9°C., and somewhat longer at 0°C. Attempts were made still further to prolong the period during which the brain would remain infective, and the following method, which has been in use for over a year, has been devised. It has been found so convenient and reliable and labour-saving that it is hoped that it may be found equally useful in the case of other viruses which are propagated by animal passages.

The method consists in placing the brain at the bottom of a large test-tube, and covering it with a volume—not greater than its own—of either pure neutral glycerin or glycerin diluted with an equal quantity of normal saline—in fact a few drops of the preservative are sufficient. A column of liquid paraffin, about 1 in. deep, is next floated on top of the glycerinated brain. The tube is kept as usual in cold storage and the glycerin is never changed subsequently.

Under these conditions the reaction of the glycerin becomes quite acid (pH 6 and under), and yet there is no obvious falling off of the infectivity of the material after 12 months. Contact with air must, therefore, have a greater deleterious effect on the survival of the virus *in vitro* than the acidity of the medium. Tables *A–F* illustrate the survival of the virus under these conditions without an appreciable loss of infectivity (except in one case, *B*) on intracerebral inoculation into normal rabbits.

	Time when tested.	Result of cerebral inoculation.
A.		
Virulent brain 597 in 50% glycerin in saline covered with 1 in. liquid paraffin (thoroughly autolysed).	75 days	+ 3 days.
	125 ,,	+ 3 ,,
	172 ,,	+ 4 ,,
	245 ,,	+ 4½ ,,
	1 year	+ 4½ ,,
B.		
Brain 618 in its own volume of 50% glycerin in saline, etc. (thoroughly autolysed).	75 days	+ 4 ,,
	125 ,,	+ 7 ,,
	172 ,,	+ 7 ,,
	245 ,,	+ 7 ,,
C.		
Brain 614 moistened with a few drops of 50% glycerin, etc. (shape and consistency fairly well preserved).	104 ,,	+ 3½ ,,
	1 year	+ 4 ,,
D.		
Brain 648 in its own volume of 50% glycerin, etc. (shape and consistency well preserved).	103 days	+ 4 ,,
	201 ,,	+ 4 ,,
	1 year	+ 5½ ,,
E.		
Brain 698 (i) moistened with a few drops of pure glycerin, etc. (well preserved ?).	6 months	+ 2½ ,,
F.		
Brain 698 (ii), no preservative added, but covered with liquid paraffin (thoroughly autolysed).	6 ,,	+ 5 ,,

Note.— + indicates death after typical clinical syndrome, presence of characteristic histological changes and recovery of the virus on passage.

In the case of *A*, *C* and *D* the strain is the one isolated from the brain of a case of encephalitis lethargica. Its usual incubation period after 26 passages since isolation two years ago has never exceeded four days so far. *B*, *E* and *F* represent a strain of herpes virus isolated from a case of labial herpes in 1922. Its normal incubation period after 70 passages varies from 4–7 days.

If intracerebral inoculations are made of portions obtained from various parts of the freshly removed brain of a rabbit which has died of a herpetic encephalitis after an intracerebral inoculation, it is not uncommon to find that the parts of the brain farthest away from the point of inoculation (*e.g.* the occipital lobes) are comparatively avirulent. The most dependent parts of the brain, on the other hand, are always highly infective wherever the site of inoculation may be. But if the brain is preserved as described above, and especially if it is thoroughly autolysed, as is usually the case when only a few

drops of the preserving fluid are used, the degree of infectivity as judged by the incubation period on intracerebral inoculation is the same for all parts of the preserved brain. The increase in the infectivity of virulent brain after the onset of autolysis has been already recorded (Perdrau, 1925), and it is usual for the incubation period to remain practically constant subsequently for many months. It is interesting to note that the virus remains quite active even if no preserving fluid is used, provided that air is excluded. This method of preservation has only been tried over a period of 6 months so far. Some samples of virulent brain have been sealed up in tubes from which all air had been excluded, and will be tested later in order to see whether the virus will survive in the complete absence of oxygen, because, as is well known, liquid paraffin only produces a relative anaerobiosis.

Among the many obvious advantages of this method of preserving virulent brain may be mentioned the great economy in animals, the saving of time and labour, and the fact that it is possible to keep the number of passages as low as one a year. The latter point seems to be a particularly important one, because Nicolau (1926) has shown that a strain of virus which has been subjected to some 700 passages in the course of six years has lost a good deal of its virulence.

The method has given quite good results in the case of virulent broth also.

MacCartney (1924) has shown that if virulent brain is added to glucose broth and the whole is incubated aerobically at 37°, the broth becomes virulent and remains so for some days—the longest period being six days.

These experiments were repeated and the broth tested after both aerobic and anaerobic incubation. More constant results were obtained with the latter, and in addition it has been found that if a layer of liquid paraffin is floated on the surface of the broth and the tube kept in cold storage after a stay of 3 to 5 days at 37°, the broth will remain infective for several weeks. In one case the broth has been found to be still infective when last tested at the 15th week.

CONCLUSION.

A method is described for the preservation of the virus of herpes which is economical in animals and time-saving, and which should be capable of preserving the virulence of the strain over considerable periods of time by limiting the number of animal passages.

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THE STANDARDIZATION OF TUBERCULIN.

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IN spite of the large amount of work that has been done on the standardization of tuberculin, we have not been able to find in the literature of the subject any adequate study of the accuracy of the tests usually employed, though this is a necessary preliminary to any legitimate claim to be able to standardize the active principle. This, no doubt, is due to the fact that at best the methods available are not very accurate, and have hardly been studied beyond the qualitative stage. In the comparison of a pair of samples of tuberculin the limitations of the tests may not be fully realized.

In the following paper we have endeavoured to collect some data which may help in determining the value of three of the best-known methods of comparison. The three tests investigated were the—

(1) Intradermic (Eagleton and Baxter, 1923),
(2) Von Pirquet (quantitative) and
(3) Subcutaneous (Frankfort, Otto and Hetsch, 1921).

In all these tests the guinea-pig was used as the experimental animal.

With regard to the intradermic and von Pirquet methods our principle was a simple one. A tuberculin of approximately the same value as the Frankfort standard was taken and compared with dilutions of itself in concentrated glycerine veal broth, which were prepared by our colleague, Dr. J. W. Trevan. It was our problem to find out what percentage differences we could detect by the various methods. Each of the dilutions was regarded by us as a tuberculin of unknown strength, and subsequently diluted appropriately in saline for the actual tests. These samples were compared with a sample of the undiluted tuberculin which was used as the standard for each experiment. A sufficient number of animals was injected so that the results would be capable of numerical analysis. After each experiment the estimated values were compared with the actual values as then supplied by Dr. Trevan.

TECHNICAL DETAILS.

The technical details of the work were on the lines indicated by Eagleton and Baxter, and need only be referred to briefly. Guinea-pigs of 300 to 350 gm. in weight were inoculated subcutaneously with 0·5 mgm. or 0·25 mgm. of a three weeks' growth of the human strain of *B. tuberculosis* (H. 12) on Dorset's medium. With this inoculum, guinea-pigs were appropriately sensitive to the intradermic and von Pirquet test in about three weeks, and to the subcutaneous test in six to eight weeks. For the intradermic test they were sensitive on an average to an injection of 0·2 c.c. of a 1 in 2000 to 1 in 4000 dilution of standard tuberculin. In the von Pirquet test they reacted to 1 in 4 to 1 in 8 of standard tuberculin. For the subcutaneous test

the guinea-pigs were sufficiently sensitive that 0·1 c.c. of standard tuberculin (= Frankfort standard) killed about 50% of the guinea-pigs tested.

The series of dilutions used in the intradermal test were usually 1 in 500, 1 in 1000, 1 in 2000 and 1 in 4000 of the preparations under test, and the amount injected 0·2 c.c. In the von Pirquet test the tuberculins were generally used undiluted, and also diluted 1 in 4, 1 in 8, 1 in 16 and 1 in 32; three parallel scratches, half an inch long, were made for each dilution. The readings were made 18 to 24 hours after injection in the case of the intradermic and von Pirquet tests. Usually the reactions were matched or graded by eye, but during part of the work measurements were taken by means of calipers, and the results almost exactly agreed with the matching by eye.

INTRADERMIC TEST IN GUINEA-PIGS.

Calmette and de Potter (1926), in their recent report on methods of preparing and standardizing tuberculin, came to the conclusion that this is the most useful method of titration. In our hands it has also proved the most valuable method.

TABLE I.—*Actual and Estimated Values of Samples of Tuberculin Tested by the Intracutaneous Method.*

Sample.	Correct values.	Estimated values.	Number of animals used.	Number of animals readable.
A	100	100	13	11
B	50	45		
C	50	55		
D	100	75		
E	100	100	10	10
F	60	70		
G	50	60	10	7
H	70	60		
J	70	65	9	7
K	40	30		
L	60	65	14	9
M	30	40		
N	70	100	9	5
O	25	35		
P	0	0	6	5
Q	10	15		
R	0	0	7	6
S	75	80		
T	10	5–20 (probably 8–10)	10	7
U	15	5–20 (probably 8–10)		
	Total	... ...	88	67

An initial attempt was made to place samples of tuberculin of different values and the standard tuberculin in their correct order. This was done with 5 series of 4 tuberculins, 50 guinea-pigs being used for the experiments. If the tuberculins differed 40% or more in potency, it was nearly always possible to place them in their correct order by the test.

Taking 40% as the difference between two tuberculins that was just clearly detectable, a further series of tuberculin preparations was compared and percentage values allotted to them (Table I). The values estimated from the test are not to be taken as more than attempts to assign a percentage, but it will be seen that the attempts were almost always good ones. Only once in 20 comparisons was the estimated value further out than 30%, and it was usually a good deal less. Considerable numbers of guinea-pigs had, however, to be used in order to arrive at this degree of accuracy, and it must be clearly understood that the reading of individual guinea-pigs may give values that are widely aberrant. For practical purposes 4 to 6 guinea-pigs will indicate differences of 40 per cent. with a fair degree of certainty, and with the use of a greater number of animals considerably finer differences may be detected. It may be added that the average standard deviation of the series of values given in Table I corresponds to an error of 22·2 per cent. of the activity of the sample tested—that is, the expected error would be rarely above 44·4%, which agrees with the rough estimate first arrived at.

QUANTITATIVE VON PIRQUET TEST IN GUINEA-PIGS.

Table II gives the results of 16 comparisons of tuberculin preparations with the standard tuberculin. It will be seen that this method gave much less satisfactory results than the intradermic method. Occasionally good estimates were made, but in many cases the estimate was very inaccurate. The chances of being able to detect 50% differences are small, and it is possible to make such a gross error as to state that a given sample is 50% of standard strength when in reality it is tuberculin-free (*e.g.* sample K1). With weak tuberculins, also, very wide errors can be made, as the maximum reactions produced on the guinea-pigs may not be good enough to be read with ease. On individual guinea-pigs the reactions due to standard tuberculin, undiluted, and diluted 1 in 4 and 1 in 8, may all be indistinguishable. To get the maximum accuracy with this test the tuberculins and their dilutions must give well-marked and well-graded reactions.

To sum up, if the tuberculins are sufficiently strong, the guinea-pigs highly sensitive, and the gradation of the reactions good, 50% differences are often detected by the von Pirquet test, but such wide errors may be made that the test may be considered as useless for the purposes of tuberculin standardization.

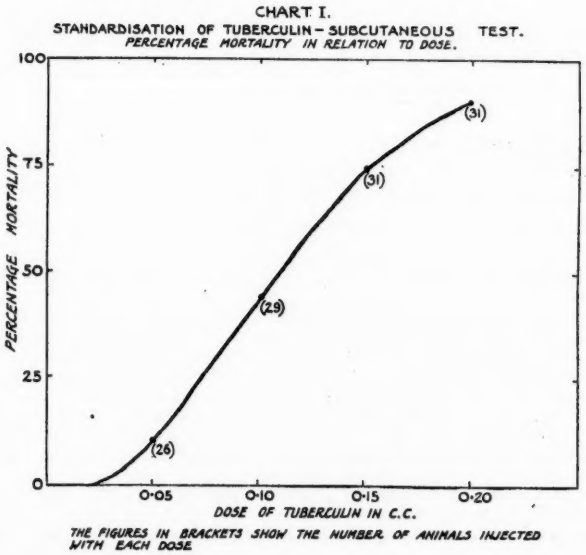
SUBCUTANEOUS TEST IN GUINEA-PIGS.

The figures for this test were arrived at by a somewhat different method. As each batch of guinea-pigs became appropriately sensitive, it was subdivided into four groups, which were injected, not with unknown dilutions, but with

TABLE II.—*Actual and Estimated Values of Samples of Tuberculin Tested by the von Pirquet Method.* (*S = Standard Tuberculin.*)

Sample.	Correct values.	Estimated values.	Number of animals used.	Number of animals readable.
V	70	100	5	4
W	80	<S		
X	50	<S<S<S=S*	9	4
Y	20	=S<S=S<S		
Z	27·5	35	7	7
A_1	10	25		
B_1	16·6	33·3	6	5
C_1	50	33·3		
D_1	0	25	5	5
E_1	50	25		
F_1	90	90	5	5
G_1	50	70		
H_1	50	25	5	5
J_1	25	12		
K_1	0	50	7	5
L_1	100	50		
Total	...	... ...	49	40

* Readings on individual guinea-pigs.



0·05, 0·1, 0·15 and 0·2 c.c. of standard tuberculin. Chart 1 represents graphically the percentage mortality in guinea-pigs in relation to increasing doses of standard tuberculin (that is to say, in relation to the actual amount of active principle present). The figures on the curve indicate the number of guinea-pigs put up at each dose. They represent several batches of tuberculous guinea-pigs, but the batches were, as far as could be arranged, of equivalent sensitiveness. From this curve it can be calculated that with two groups of eight animals, tuberculins in an activity ratio of 2 to 1 (*i.e.* a drop of 50%) will not be placed in the wrong order more than once in 21 times. On this calculation it would require 25 animals in each group to differentiate a sample of tuberculin from one 30% less potent.

CHART II.

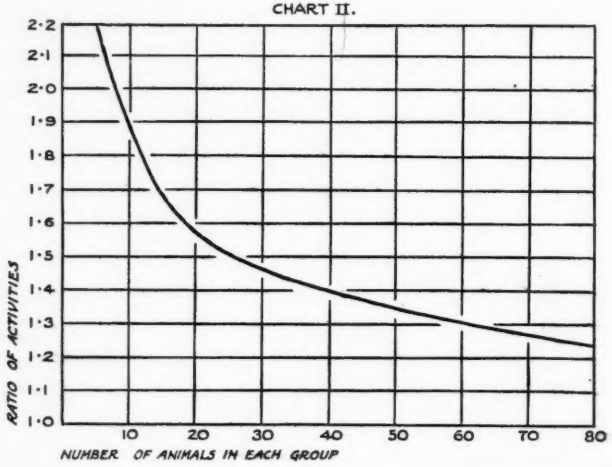


Chart 2, kindly constructed for us by Dr. Trevan, utilizes the same data to show the number of animals required to obtain a given degree of accuracy. Thus to distinguish 20 times out of 21 a tuberculin of value 1·4 from a tuberculin of value 1 it would require 40 guinea-pigs for each tuberculin, and so on.

We are indebted to Dr. J. W. Trevan for the following note:

The curve (Chart 2) shows approximately the various ratios of the doses of tuberculin which are sufficient to give a significant difference in mortality using various numbers of animals. The number of animals injected with each dose is given by the abscissæ, and the ratios given by the curve are the ratios corresponding to twice the standard deviation of the difference of the mortality in the two groups. The ratios given therefore represent doses of which the smaller will give a mortality less than the larger, in 20 cases out of 21 on the average. The curve has been calculated for the cases in which the doses administered happened to be such that the mortalities are symmetrically disposed around 50% mortality. For other pairs of mortalities the ratio giving a significant difference may be slightly different. The values of the ratio for 40 animals, for example, may fluctuate between 1·36 and 1·56. The curve was constructed in the following manner:

Let S be the standard deviation of the difference of mortality in the 2 groups; then

$2S$ represents a significant difference. If the doses are such that the mortalities are symmetrically disposed around 50 %,

$$S = 100\sqrt{\frac{2p\,(1-p)}{N}}$$

expressed as % mortality, where p is the probability of death with the smaller and survival with the larger dose and N is the number of animals in each group and—

$$\frac{100-2S}{2} = 100p,$$

and from this it can be shown that—

$$p = \tfrac{1}{2} \pm \sqrt{\frac{1}{2N+4}}.$$

$100p$ is the percentage rate of mortality given by one dose and $100\,(1-p)$ is the percentage rate of mortality just significantly different. The doses which correspond to these mortalities are read from the mortality curve (Chart 1) and the ratio calculated for different values of N.

It is of course clear that all these calculations depend on the accuracy with which the curve (in Chart 1) represents the true relationship between mortality and dose. The number of observations is not large and there is a possibility of error in the estimates of the ratios calculated from it, but the calculations give an idea of the order of accuracy of the test.

SUMMARY.

The intradermic test is more accurate than the subcutaneous or von Pirquet methods, differences representing a 40% drop being almost always detectable on a small series, and finer differences on a larger series of guinea-pigs. The test, however, calls for considerable experience.

The subcutaneous test will also show differences of 40–50%, if a sufficient number of suitable guinea-pigs is used, but the cost in guinea-pigs of attaining a given degree of accuracy is much greater than with the intradermic test. Its one advantage is that there is practically no personal factor in its interpretation.

The von Pirquet test in guinea-pigs is very inaccurate and gross errors are often made. It is practically useless as a method of standardizing tuberculin.

We have to thank our colleague, Dr. J. W. Trevan, for much help and criticism in this work.

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THE ACTION OF TUMOUR EXTRACTS ON TISSUES *IN VITRO*.

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It has already been shown that autolysed extracts of adult tissues and non-autolysed extracts of various tumours possess the power of producing active proliferation of tissues *in vitro*, many tumour fragments becoming detached and growing actively over the slide (Drew, 1923; Erdmann, 1926). These tumour extracts, though usually far more potent than autolysed extracts of normal tissues, were often found to be very erratic, different extracts of the same strain of tumour, but prepared at various times, sometimes giving a powerful extract, and at others being inert. Heaton (1926) re-investigated the nutritive requirements of growing cells *in vitro*. He rightly distinguished between real growth, *i. e.* multiplication of cells, and mere survival, viz. emigration. Working with embryonic chick heart, liver and intestine in my saline medium (Drew, 1922), he found that these tissues would grow without the addition of any other substances provided they were taken from embryos not older than 11 days. After this period the tissues behaved like adult ones, and growth could not be obtained without the addition of embryo extract, or certain extracts of various organs, or of yeast. He further showed that 97% alcohol extracted from yeast some substance which promoted the growth of epithelia if added to cultures in the saline medium. In addition he described an inhibitant to fibroblasts which was extracted from yeast by 75% alcohol. Both these extracts were thermostable.

These experiments of Heaton's suggested that if both a stimulant and an inhibitory body was present in the same extract, it might afford an explanation of the erratic behaviour of various tumour extracts. It was decided to investigate Heaton's claim, on chick and mammalian tissues, heart and kidney being generally the tissues used.

A quantity of Merck's dried yeast was ground up and extracted with 97% alcohol, a further quantity being extracted with 75% alcohol. The extracts were filtered through paper and evaporated to dryness *in vacuo*. The residue was then taken up in a small volume of the saline medium filtered through paper and then through a Berkefeld candle. Cultures of heart and kidney from 15-day-old chicks were put up in the following media:

(1) Saline alone.
(2) 3 parts saline + 1 part of embryo extract.
(3) 3 parts saline + 1 part of 97% alcohol-yeast extract.
(4) 3 parts saline + 1 part of 75% alcohol-yeast.

Results are shown in following tables:

Heart (15 days).

No.	Medium.	Degree of growth. Time in days. 1.	2.	3.	4.	5.
1	Saline only	...	...	...	...	...
2	,,	...	...	...	...	Trace
3	,,	...	...	...	...	...
4	,,	...	...	...	...	...
5	,,	...	...	...	...	...
6	,,	...	...	...	...	...

Kidney (15 days).

1	3 parts saline + 1 part embryo extract	+	++	...	...	...
2	Ditto	+	+++	...	...	...
3	,,	+	++	...	...	...
4	,,	Trace	++	...	...	...
5	,,	...	+	...	...	...
6	,,	+	+++	...	...	...

Kidney (15 days).

1	3 parts saline, 1 part 97% alcohol-yeast extract	+	+	++	...	...
2	Ditto	+	++	+++	...	...
3	,,	Trace	++	++	...	...
4	,,	,,	+	++	...	...
5	,,	+	++	+++	...	...
6	,,	+	++	+++	...	...

Heart.

No.	Medium.	Degree of growth. Time in days. 1.	2.	3.	4.
1	3 parts saline, 1 part 97% alcohol-yeast extract	...	...	...	...
2	Ditto	...	...	Trace	Trace
3	,,	...	...	...	...
4	,,	...	...	...	...
5	,,	...	...	Trace	Trace
6	,,	...	...	...	...

Heart.

No.	Medium.	Degree of growth. Time in days. 1.	2.	3.	4.
1	3 parts saline, 1 part 75% alcohol-yeast extract	...	...	...	...
2	Ditto	...	...	...	...
3	,,	...	...	...	...
4	,,	...	...	...	...
5	,,	...	...	...	...
6	,,	...	...	...	...

Kidney.

1	3 parts saline, 1 part 75% alcohol-yeast extract	...	Trace	+	+
2	Ditto	...	...	...	...
3	,,	...	...	...	...
4	,,	...	...	...	...
5	,,	...	...	...	...
6	,,	...	Trace	Trace	Trace

It seems clear from these experiments that Heaton's contention is correct, viz. that 97% alcohol extracts from yeast a substance which promotes the growth of epithelia, whilst 75% alcohol extracts an inhibitant to fibroblasts which is indifferent to epithelia. Similar experiments with mammalian heart and kidney gave similar results. As Heaton has pointed out, various samples of yeast differ greatly in their potency, some being relatively inert. We have found Merck's to give by far the best results.

Embryonic rat or mouse heart and kidney cultivated in saline gave growth only, but if taken from embryos nearly at term, they failed to grow without the addition of embryo extract. Tumours in many respects behave as embryonic tissues in culture, so that it was important to ascertain if they also followed young embryonic tissues in their capability to grow in the plain saline fluid. Cultures of the following tumours were put up in the plain saline medium and also in saline medium + $\frac{1}{3}$ of its volume of embryo extract: 37 sarcoma, 63 carcinoma, 206 carcinoma, J.R.S. sarcoma.

No growth took place at all in the plain saline cultures, the tumours behaving exactly as if they were adult tissues. The cultures in saline + embryo extract, however, showed growth in the usual way. That tumours should behave as do adult tissues is perhaps not surprising as they are derived from adult cells.

It was next decided to endeavour to ascertain whether tumours contained the alcohol-soluble epithelial stimulant and fibroblast inhibitant that had been described by Heaton for yeast and other tissues.

Three tumours were used for this purpose—a sarcoma 37S, and a mammary carcinoma 63 and a rat sarcoma J.R.S. A number of tumours were removed and finely minced and finally ground up with sand. The mass was then extracted with 97% and 75% alcohol and filtered. The alcohol was removed by evaporation under reduced pressure and the resulting extracts dissolved in

saline medium. The extracts were then tested in culture with heart, kidney, 37S and 63, and in no case were they found capable of promoting growth. Plain saline extracts of these tumours promoted growth in the manner already described (Drew, 1923). The stimulant substance in tumour extracts is therefore not of the same nature as these growth-promoting substances found in alcoholic extracts of yeast and various organs. This of course was anticipated, as Heaton describes these bodies as being thermostable, whilst heating at 56°C. quickly destroys the activity of tumour extracts. Working with numbers of saline extracts prepared from the same tumour strain, it became apparent that a tumour like 63 or 206 which generally gave a powerful epithelial stimulant, at times gave an extract which was not only inert, but contained an inhibitory substance. This point is well brought out in the two following charts of experiments:

No.	—	—	Degree of growth. Time in days. 1.	2.	3.
2219	Kidney	3 parts saline + 1 part embryo extract + 1 part 206 extract No. 2	Trace	+	++
2220	,,	Ditto	+	++	+++
2221	,,	,,	++	+++	++++
2222	,,	,,	++	+++	+++
2223	,,	,,	+	++	++++
2224	,,	,,	++	+++	+++
2230	Kidney	3 parts saline + 1 part embryo extract + 1 part 206 extract No. 3	...	...	...
2231	,,	Ditto	...	...	...
2232	,,	,,	...	...	...
2233	,,	,,	...	...	...

Portions of embryo kidney were put up in media differing only in the tumour extract. It will be seen that extract No. 2 stimulates growth very readily, whilst extract No. 3 inhibits growth. Both extracts were prepared in an identical manner, and the only difference was that extract No. 2 was prepared from a tumour that was actively growing, whilst extract No. 3 was from one during a depression period.

Similar experiments with other tumour extracts as J.R.S. and 37S showed the same thing, extracts prepared from tumours which were proliferating rapidly yielding active extracts, whilst those taken from tumours at the onset of, and during a depression period, gave extracts that had a directly inhibiting action.

Satisfactory extracts from tumours require careful preparation in order to obtain uniform results. The method adopted in all these experiments has been to remove the tumour aseptically and grind up with sand in an ice-cooled mortar with approximately its own volume of the saline medium. The mixture is then rapidly centrifuged and the supernatant fluid filtered through a Berkefeld candle. Cultures are put up in a mixture of saline 3 parts and tumour extract 1 part. The pH of the final mixture has been adjusted in

these experiments to 7·3. In all cases the extracts when prepared from carcinomata appeared to have a definitely stimulant action on epithelial tissues, whilst the sarcoma extracts, though giving an increased fibroblast growth, appeared inert towards epithelial tissues generally. It appears that during depression periods tumours not only do not produce a stimulant to growth, but elaborate some substance or substances that are definitely inhibitory in a similar manner to that described by Carrel and Ebeling in the case of tissues derived from old animals. If tumours are carefully selected from young rapidly growing strains good extracts can be obtained. There appears to be a fundamental difference between the growth-promoting factors in embryo extract and tumour extracts, and those bodies derived by Heaton from alcoholic extracts of yeast and other tissues.

The suggestion has been made that a great deal of the stimulating effects of various autolysed tissue extracts and tumour extracts is due to alteration of the pH of the medium. That this view was untenable was long ago showed by Carrel, Ebeling and Fischer. Maximum growth takes place between the comparatively narrow limits of 6·8 to 7·8. In my experiments the optimum growth was obtained with a pH of 7·3 to 7·4. Numerous experiments have been made with variations of pH, but in no case were results obtained comparable to those with extracts. The following tables illustrate some of these experiments. It will be noticed that where the pH of the cultivating fluids is adjusted to the same value, the amount of growth is greater in media containing autolysed extract. Variation of the pH from 6·3 up to 7·8 always gave greater growth in those fluids containing a tissue extract. In making the necessary adjustment, the acid or alkali should be sufficiently concentrated so as to give the required pH by the addition of a drop or two only, as otherwise the tonicity of the medium is affected, and it is well known that an increased growth of tissues is obtained if the medium is rendered hypotonic by the addition of distilled water. pH estimations of the small quantities of fluid used in *in vitro* cultivation are conveniently made with the capillator supplied by the British Drug Houses. Lactic acid and KHO are convenient for effecting the desired alterations in pH.

No.	Date.	Tissue.	Medium.	Degree of growth. Time in days. 1.	2.	3.
1272	11.11.26	Chick embryo, heart	Saline medium 1, embryo extract 2, autolyzed tissue extract 1; pH adjusted to 7·3	Trace	++	...
1274	,,	Ditto	Ditto	++	+++	...
1275	,,	,,	,,	+	++	...
1276	,,	,,	,,	++	+++	...
1277	,,	,,	,,	++	+++	...
1278	,,	,,	,,	+	+	...
1279	,,	,,	,,	++	++	...
1280	,,	,,	,,	++	+++	...
1281	,,	,,	,,	Trace	+	...

No.	Date.	Tissue.	Medium.	Degree of growth. Time in days. 1.	2.	3.
1282	11.11.26	Chick embryo, heart	Saline 1, embryo extract 2; pH adjusted to 7·3	...	...	...
1283	,,	Ditto	Ditto	...	+	...
1284	,,	,,	,,	...	...	...
1285	,,	,,	,,	...	Trace	...
1286	,,	,,	,,	...	,,	...
1287	,,	,,	,,	...	...	...
1288	,,	,,	,,	...	+	...
1289	,,	,,	,,	...	Trace	...
1290	11.11.26	Chick, heart	Saline 1, embryo extract 2; adjusted to pH 6·5	...	...	...
1291	,,	Ditto	Ditto	...	...	...
1292	,,	,,	,,	...	...	...
1293	,,	,,	,,	...	...	...
1294	,,	,,	,,	...	Trace	...
1295	,,	,,	,,	...	...	...
1296	,,	,,	,,	...	Trace	...
1297	,,	,,	,,	...	...	...
35	3.10.26	Kidney (mouse)	Saline 3, embryo extract 1; pH 7·2	...	Trace	++
36	,,	Ditto	Ditto	...	...	+
37	,,	,,	,,	...	Trace	+
38	,,	,,	,,	...	...	Trace
39	,,	,,	,,	...	...	...
40	,,	,,	,,	...	+	++
41	,,	,,	,,	...	Trace	+
42	,,	,,	,,	...	...	+
43	3.10.26	Kidney (mouse)	Saline 3, embryo extract 1, autolyzed tissue extract 1; pH 7·2	+	++	+++
44	,,	Ditto	Ditto	Trace	++	+++
45	,,	,,	,,	+	+	++
46	,,	,,	,,	+	++	+++
47	,,	,,	,,	...	++	+++
48	,,	,,	,,	Trace	++	++

No.	Date.	Tissue.	Medium.	Degree of growth. Time in days. 1.	2.	3.
1214	3.10.26	Kidney (mouse), 3 days old	Saline 3, embryo extract 1; pH adjusted to 6·4	...	...	Trace
1215	,,	Ditto	Ditto	...	...	...
1216	,,	,,	,,	...	...	...
1217	,,	,,	,,	...	...	...
1218	,,	,,	,,	...	...	...
1219	,,	,,	,,	...	...	Trace
1220	3.10.26	Kidney (mouse), 3 days old	Saline 3, embryo extract 1; pH 6·5	...	...	Trace
1221	,,	Ditto	Ditto	...	...	,,
1222	,,	,,	,,	...	...	...
1223	,,	,,	,,	...	Trace	+
1224	,,	,,	,,	...	...	...
1225	,,	,,	,,	...	...	...
1226	3.10.26	Kidney (mouse), 3 days old	Saline 3, embryo extract 1; pH. 6·8	...	Trace	+
1227	,,	Ditto	Ditto	...	...	Trace
1228	,,	,,	,,	...	...	+
1229	,,	,,	,,	...	...	+
1230	,,	,,	,,	...	Trace	+
1231	,,	,,	,,	...	,,	+
1232	,,	,,	,,	...	...	+
1233	,,	,,	,,	...	...	...
1234	,,	,,	,,	...	...	Trace

SUMMARY.

The work of Heaton on the action of alcoholic yeast extracts on growth in tissue cultures is confirmed.

Alcoholic extracts of transplantable mouse tumours prepared in the same way as the yeast extracts were without effect on growth.

The watery extracts from transplantable tumours varied in activity according as they were prepared from tumours growing rapidly or from receding tumours.

The action of the watery extracts is not due to alterations in hydrogen-ion concentration.

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ON THE CONSTITUTION OF DYSENTERY TOXIN AND ITS RELATION TO THE TESTING OF ANTIDYSENTERY SERUM.

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From the Lister Institute, Elstree, Hertfordshire.

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THE work of the delegates to the International Conferences (1924, 1926) on the testing of antidysentery (Shiga) serum has helped considerably towards establishing an adequate method of titration. All are now agreed that a standard serum is the essential requisite. This serum has been prepared in Dr. Madsen's laboratory in Copenhagen, and along with it there has been issued a standard dry toxin consisting of powdered bodies of the dysentery bacillus. A provisional unit, which in fact represents a definite amount of the standard serum, has been established. These are important steps in the direction of a workable method of titration, and yet the actual technique is beset with difficulties that seem to be peculiar to this toxin. For example, irregularities in the tests, whether on rabbits or mice, are often alluded to as adding to the difficulties of titration; discussions on the merits of the mouse or rabbit as the test animal are probably referable to this circumstance. At least six kinds of toxin preparations may be used as test toxins; it is, however, by no means certain that they are of equal suitability for purposes of titration. One of these—the dried bacillary product—has the advantage of remaining of constant value over long periods. For example, the toxicity of one prepared by Dr. A. T. MacConkey in this laboratory was unchanged after nine and a half years. As to the stability of broth toxins, contradictory statements have been made.

Lastly, a matter of basal importance in standardization tests is still undecided, namely, whether dysentery toxin is a simple homogeneous toxic substance, or whether, like diphtheria toxin, it consists of a mixture of the lethal toxic substance and of an atoxic derivative, or toxoid, which, however, is still capable of binding the specific antitoxin. On this question different workers hold opposite views.

Disadvantages inherent in the use of dry bacillary toxins led me to examine the suitability of broth toxins for testing purposes, and at the same time to inquire into the question of the existence of toxoids.

THE METHOD OF PREPARATION OF THE BROTH TOXINS INVESTIGATED.

On 30.4.26 eighteen one-litre bottles each containing 500 c.c. of the same batch of Berna-peptone broth with a reaction of pH 7·8 were inoculated with each of our stock strains of the Shiga dysentery bacillus. The cultures were incubated at 37° C for 5 weeks—that is, till 5.6.26. On this date the bottles were removed from the hot room, and the cultures, after the addition of

toluol, were left at room temperature for 48 hours. After removing a 50 c.c. sample from each bottle the cultures were mixed, and a 50 c.c. sample was again taken. The bacilli were filtered off from the mixed batch by means of a Seitz filter, and the resulting toxic filtrate was treated with formalin and used to immunize a horse, "May," as described below.

The toxins were prepared from the toluolized samples at the time of testing by centrifuging them and pipetting off the supernatant fluid from the bacterial deposit. Filtration through a kieselguhr, porcelain or asbestos filter would have been preferable, but it was hoped in the first instance to obtain sufficient data to serve as a basis for observations with filtered toxins.

The results with four other toxins are available for comparison:

(1) Toxin No. 222, a filtered broth toxin;
(2) A sulphated toxin, No. 203, prepared in this laboratory by Dr. A. T. MacConkey;
(3) The first dry standard toxin issued from Dr. Madsen's laboratory; and
(4) A dry bacillary toxin prepared at the Pasteur Institute, Paris.

Toxin No. 222.—A three weeks old broth culture of Strain K. 624 was filtered through a kieselguhr filter and the filtrate was kept under toluol in the cold room.

Toxin No. 203.—Six strains of the Shiga bacillus were grown on a series of batches of agar in bottles for 2 to 5 days at 37° C.; the growths were then washed off in chloroformed distilled water, and were allowed to autolyse from 11 to 33 days in the cold room. On 11.12.22 the autolysates were mixed and filtered through a kieselguhr filter. Next day anhydrous sodium sulphate was added to saturation at 37° C., the precipitate was filtered off, dried, and ground in a mortar to a fine powder. This toxin contains 32·9% of Na_2SO_4, and is almost completely soluble in water.

The Copenhagen dry standard toxin.—An agar emulsion was killed by heating to 58° C. for 20 minutes, and the bacilli were spun, dried in a desiccator and ground to a powder.

The Pasteur Institute dry toxin, "I.P."—This toxin was prepared from Strain No. 30. The bacterial emulsion was not heated before drying.

THE METHOD OF CARRYING OUT THE TESTS.

The M.L.D. of the several toxins was determined by intravenous injection of 15–20 gm. mice, and of rabbits whose average weight was 1200 gm. (900–1500 gm.). The period of observation was 7 days. The L_+ and L_o doses of each toxin were tested on mice by means of a constant quantity, namely, 1/300 c.c. of a 1% solution of the Copenhagen standard dried serum. For convenience of description and comparison the term L_+ dose is used here to indicate the minimum dose of toxin given in these tests, which, when injected intravenously into mice of 15–20 gm. weight together with 1/300 c.c. of the standard serum after mixture and contact for three-quarters of an hour at 37° C., killed a mouse within 7 days; and the L_o dose as the greatest amount of toxin which, when injected together with 1/300 c.c. of the standard serum under the same conditions, allowed the mouse to survive beyond the 7th day.

In each test on any particular day one animal only—mouse or rabbit—was used for each dose, but extensions or repetitions of the tests were occasionally carried out at a later date. The protocols of the mouse tests are given in Table I, and the results of both mouse and rabbit tests are arranged in Table II.

One only out of the 18 strains failed to give evidence of toxin, *i.e.* sample No. 2; intravenous injection of 1/10th c.c. did not kill a rabbit. This sample was not further investigated.

THE DEGREE OF REGULARITY OF THE TESTS.

The tests on mice are set forth in Table I, and show on the whole a fair degree of regularity. Those of toxins 1, 4, 17, 225 and 222 suggest, indeed, a graduated relationship between dose and death-time. Zangger (1923) has stated that both for rabbits and mice the interval between injection and death certainly lengthened with falling doses, but that the deviations from the rule were so numerous as to exclude the factor of death-time from the definition of the lethal dose. This relationship is much less likely to be evident in tests on rabbits, because most of the deaths take place within three days of the inoculation. Thus, out of 61 deaths noted in determining the M.L.D. for rabbits (see Table II), 42 occurred within the first 3 days, 7 between the third and fourth day, that is, they died during the night, and 12 later than the fourth day. Apart from this result the M.L.D. tests on rabbits were more irregular than the mouse tests, so that repetitions of the tests on this account were more often necessary in rabbits than in mice. We have no set of similar data for a dry bacillary toxin on mice, but the comparison is worth making, and so it was thought worth while to publish the protocols in full. In this connection we may note that the samples which yielded a clear supernatant fluid after spinning gave on the whole more regular results than those which yielded a slightly cloudy supernatant fluid.

Various explanations of the irregularities in the mouse test for dysentery have been given, and it is generally accepted that the mouse possesses a varying susceptibility to the toxin. There is one aspect of the subject which seems to deserve more attention than it has received—O'Brien (1924, 1925) alone of the authorities has touched upon it—namely, the importance of insuring as far as possible that the mice at the time of test are healthy, and that they have been derived from a uniform breeding stock whose welfare is carefully followed from day to day. Experience here has shown that it is unsafe to depend on imported stock, and that if mice from an unhealthy stock be used the tests will show a high degree of irregularity. We make a rule to examine every mouse that dies, not only among the test animals, but among the breeding stock from which they are taken. With mice, as with guinea-pigs, the winter is the season in which the mortality is highest and the risk of epidemics greatest. Not only do chronic infections precipitate death from dysentery toxin, but in view of recent work on the reticulo-endothelial system from the standpoint of immunity, it may also be that in some instances the mouse has a heightened resistance to the toxin owing to a recent or existing infection.

TABLE I.—*Protocols of M.L.D. and L_+ Tests of Liquid Dysentery Toxins.*

The sign "+" = death; the sign "S" = survival. The data are arranged in order of the dose of toxin given and not in order of the date of testing. "C.S.S." = Copenhagen standard serum.

Toxin No.	Weight of mouse.	Dose (c.c.).	Number of M.L.Ds. in test dose.	Day of experiment. 1.	2.	3.	4.	5.	6.	7.	Date of test.
1	20	0·2	...	+	...	...	...	...	...	...	25.6.26
	20	0·1	...	...	...	+	...	...	...	...	„
	20	0·04	...	...	...	...	+	...	..	...	„
	15	0·02	...	...	...	...	...	...	...	+	22.7.26
	18	0·0133	...	...	...	...	...	...	...	S	7.9.26
1	24	1/300 C.S.S. +0·8	40	+	...	...	...	...	...	...	17.9.26
	22	„ „ +0·7	35	...	...	+	...	...	...	...	„
	20	„ „ +0·6	30	...	...	+	...	...	...	...	„
	18	„ „ +0·5	25	...	...	...	...	...	...	+	„
	18	„ „ +0·4	20	...	...	...	...	...	...	S	27.9.26
	20	„ „ +0·3	15	...	...	...	...	...	...	S	„
3	15	0·02	...	...	+	...	...	...	...	...	16.7.26
	15	0·0133	...	...	...	...	+	...	...	...	„
	24	0·01	...	...	...	...	...	+	...	...	22.7.26
	15	0·0066	...	...	...	...	...	...	...	S	„
3	20	1/300 C.S.S. +0·32	40	...	...	..	+	...	...	...	17.9.26
	20	„ „ +0·28	35	...	...	...	+	...	...	...	„
	18	„ „ +0·24	30	...	...	...	...	...	...	S	„
	18	„ „ +0·20	25	+	...	...	...	...	...	...	„
4	15	0·1	...	+	...	...	...	...	...	...	22.7.26
	15	0·04	...	+	...	...	...	...	...	...	„
	15	0·02	...	...	...	...	+	...	...	...	„
	15	0·0133	...	...	+	...	...	...	...	...	7.9.26
	20	0·01	...	...	...	...	...	...	...	+	„
	20	0·0066	...	...	...	...	...	...	...	S	„
4	20	1/300 C.S.S. +0·40	40	+	...	...	...	...	...	...	17.9.26
	20	„ „ +0·35	35	+	...	...	...	...	...	...	„
	18	„ „ +0·30	30	...	+	...	...	...	...	...	„
	20	„ „ +0·25	25	...	...	+	...	...	...	...	„
	23	„ „ +0·20	20	...	...	...	...	+	...	...	27.9.26
	19	„ „ +0·15	15	...	...	...	...	...	+	...	„
5	16	0·02	...	...	...	...	+	...	...	...	22.7.26
	15	0·0133	...	...	...	...	+	...	...	...	„
	15	0·01	...	...	...	+	...	...	...	...	„
	18	0·0066	...	...	...	...	...	...	...	+	7.9.26
	17	0·005	...	...	...	...	...	...	...	+	„
	15	0·004	...	...	...	...	...	...	+	...	14.9.26
	19	0·0033	...	...	...	...	...	...	...	+	27.9.26
	19	0·0025	...	...	...	...	...	...	...	S	„

TABLE I—*continued.*

Toxin No.	Weight of mouse.	Dose (c.c.)	Number of M.L.Ds. in test dose.	Day of experiment. 1.	2.	3.	4.	5.	6.	7.	Date of test.
6	22	0·02	...	...	...	...	...	+ ...	...	...	25.6.26
	16	0·0133	...	...	...	...	...	...	...	+ ...	„
	18	0·01	...	...	...	...	...	...	+ ...	...	„
	15	0·01	...	...	...	...	+	...	...	..	3.8.26
	15	0·0066	...	...	...	...	...	...	...	S	22.7.26
	15	0·005	...	...	...	...	...	...	...	+ ...	„
	15	0·005	...	...	...	...	...	...	...	+	3.8.26
	16	0·0033	...	...	...	...	...	...	...	S	22.7.26
	15	0·0033	...	..	...	...	...	...	...	S	3.8.26
6	15	1/300 C.S.S. + 0·40	80	...	...	...	+ ...	...	...	...	3.8.26
	15	„ „ + 0·35	70	...	...	...	...	...	...	+ ...	„
	18	„ „ + 0·30	60	...	...	...	...	...	...	S	19.7.26
	18	„ „ + 0·25	50	...	...	...	...	...	...	S	„
	22	„ „ + 0·25	50	..	...	...	...	...	...	S	17.9.26
	18	„ „ + 0·20	40	...	...	...	...	...	...	S	19.7.26
	15	„ „ + 0·20	40	...	...	...	...	...	...	S	17.9.26
	20	„ „ + 0·17	35	...	...	...	...	...	...	S	„
	18	„ „ + 0·15	30	...	...	...	...	...	...	S	„
	20	„ „ + 0·125	25	...	...	...	...	...	...	S	„
7	22	0·0133	...	...	...	...	...	+ ...	...	...	16.7.26
	18	0·01	...	...	...	...	...	...	+ ...	...	„
	19	0·0066	...	...	...	...	...	+ ...	...	...	7.9.26
	16	0·005	...	...	...	...	+	...	...	...	14.9.26
	16	0·004	...	...	...	...	...	...	...	S	„
8	19	0·04	...	...	...	...	+ ...	...	..	...	7.9.26
	15	0·02	...	...	...	...	...	...	...	S	„
9	15	0·02	...	...	+	...	...	...	...	...	28.6.26
	15	0·0133	...	... +	...	...	...	...	...	...	1.7.26
	15	0·01	...	...	...	...	...	...	...	+	„
9	17	1/300 C.S.S. + 0·40	40	...	...	...	...	+ ...	...	...	17.9.26
	20	„ „ + 0·35	35	...	...	...	...	...	...	... +	„
	15	„ „ + 0·30	30	...	...	...	...	...	+ ...	...	„
	17	„ „ + 0·25	25	...	...	...	...	...	...	S	„
10	20	0·0133	...	...	...	...	...	...	...	+ ...	1.7.26
	18	0·01	...	...	...	...	...	+ ...	...	...	„
	21	0·0066	...	...	...	..	...	...	+ ...	...	16.7.26
	20	0·005	...	...	...	...	...	...	+	...	„
	15	0·004	...	...	...	...	...	...	...	S	7.9.26
10	18	1/300 C.S.S. + 0·30	60	... +	...	...	...	...	...	...	19.7.26
	15	„ „ + 0·25	50	...	...	...	...	...	...	+ ...	„
	16	„ „ + 0·20	40	...	...	...	...	...	...	+	„
	20	„ „ + 0·20	40	...	...	...	...	...	...	...	+ 17.9.26
	18	„ „ + 0·17	35	...	...	...	...	...	...	S	„
	17	„ „ + 0·15	30	...	...	...	...	...	...	S	„
	20	„ „ + 0·125	25	...	...	...	...	...	...	S	„

TABLE I—*continued.*

Toxin No.	Weight of mouse.	Dose (c.c.)	Number of M.L.Ds. in test dose.	Day of experiment. 1.	2.	3.	4.	5.	6.	7.	Date of test.
11	20	0·02	...	...	...	...	...	+	...	...	25.6.26
	21	0·0133	...	...	...	...	...	...	+	...	„
	22	0·01	...	...	...	...	...	...	+	...	„
	15	0·0066	...	...	...	...	...	...	...	S	7.9.26
	15	0·0066	...	...	...	...	...	..	...	...	+ 14.9.26
	15	0·005	...	...	...	..	...	+ ...	...	...	7.9.26
	15	0·005	...	...	...	...	...	+ ...	...	...	14.9.26
11	15	1/300 C.S.S. + 0·30	60	...	...	...	...	...	+	...	19.7.26
	15	„ „ + 0·25	50	...	...	...	...	...	+ ...	...	„
	17	„ „ + 0·20	40	...	...	...	....	...	...	S	„
12	15	0·01	...	...	+	...	...	...	...	...	28.6.26
	15	0·0033	...	...	...	...	...	...	...	+ ...	„
	18	0·0025	...		...	...	...	...	...	+ ...	7.9.26
	18	0·002	...	...	...	...	...	...	...	S	„
12	18	1/300 C.S.S. + 0·15	60	...	...	+ ...	...	...	...	...	19.7.26
	19	„ „ + 0·125	50	...	...	...	...	...	+ ...	...	„
	17	„ „ + 0·10	40	...	...	...	+	...	...	...	„
	15	„ „ + 0·075	30	...	...	...	...	...	...	...	+ 3.8.26
	15	„ „ + 0·05	20	...	...	...	...	...	...	S	„
13	20	0·0133	...	...	...	+	...	...	...	...	1.7.26
	15	0·01	...	...	...	+	...	...	...	...	„
	24	0·0066	...	...	...	...	...	+	...	...	16.7.26
	19	0·005	...	...	...	+ ...	...	...	...	...	„
	15	0·004	...	...	+ ...	...	...	...	...	...	7.9.26
	18	0·0033	...	...	...	...	...	...	...	S	„
13	15	1/300 C.S.S. + 0·30	75	+	...	...	...	...	...	...	19.7.26
	15	„ „ + 0·25	62	...	+ ...	...	...	...	...	...	„
	15	„ „ + 0·20	50	+	...	...	...	...	...	...	17.9.26
	18	„ „ + 0·16	40	+	...	...	...	...	...	...	„
	20	„ „ + 0·14	35	...	+ ...	...	...	...	...	...	„
	20	„ „ + 0·12	30	...	...	...	+ ...	...	...	...	„
	19	„ „ + 0·10	25	...	...	...	+ ...	...	...	...	„
14	15	0·02	...	...	...	+ ...	...	...	...	...	16.7.26
	17	0·0133	...	...	...	...	...	...	+ ...	...	„
	21	0·01	...	...	...	+	...	...	...	...	„
	15	0·0066	...	...	...	...	+ ...	...	...	...	7.9.26
	15	0·005	...	...	...	...	+ ...	...	..	...	„
	17	0·004	...	+	...	...	...	...	...	...	„
	15	0·0033	...	...	...	...	+ ...	...	...	...	14.9.26
	19	0·0025	...	...	...	...	...	...	...	S	27.9.26
15	20	0·02	...	...	...	...	...	+ ...	...	...	16.7.26
	19	0·0133	...	...	...	...	+ ...	...	...	...	„
	18	0·01	...	...	...	...	...	+ ...	...	...	„
	15	0·008	...	...	...	...	...	...	...	S	7.9.26
	18	0·0066	...	...	...	...	...	...	...	S	„

TABLE I—*continued.*

Toxin No.	Weight of mouse.	Dose (c.c.)	Number of M.L.Ds. in test dose.	Day of experiment. 1.	2.	3.	4.	5.	6.	7.	Date of test.
15	20	1/300 C.S.S. + 0·40	40	... +	...	...	...	...	...	...	17.9.26
	19	„ „ + 0·35	35	...	...	...	... +	...	...	...	„
	20	„ „ + 0·30	30	...	...	...	...	...	...	... +	„
	18	„ „ + 0·25	25	...	...	... +	...	...	...	...	„
	17	„ „ + 0·20	20	...	...	...	...	...	...	S	27.9.26
	15	„ „ + 0·15	15	...	...	...	...	...	...	S	„
16	17	0·02		...	...	...	...	+	...	...	16.7.26
	15	0·0133		...	...	+	...	...	...	...	„
	16	0·01		...	...	...	... +	...	...	...	„
	15	0·008		...	...	...	...	...	...	+	7.9.26
	15	0·0066		...	...	...	...	...	...	... +	„
16	21	1/300 C.S.S. + 0·32	40	+	...	...	...	...	...	...	17.9.26
	22	„ „ + 0·28	35	... +	...	...	...	...	...	...	„
	18	„ „ + 0·24	30	...	...	...	...	...	...	... +	„
	20	„ „ + 0·20	25	...	...	...	... +	...	...	...	„
	18	„ „ + 0·16	20	...	...	...	...	...	...	S	27.9.26
	15	„ „ + 0·12	15	...	...	...	...	...	...	S	„
17	18	0·2		...	...	...	...	...	...	...	25.6.26
	18	0·1		...	...	+	...	...	...	...	„
	20	0·04		...	...	...	... +	...	...	...	„
	15	0·02		...	...	...	...	... +	...	...	14.9.26
	15	0·0133		...	...	...	...	...	...	S	„
	15	0·01		...	...	...	...	...	...	S	„
18	15	0·02		...	+	...	...	...	...	...	16.7.26
	15	0·0133		...	... +	...	...	...	...	...	„
	15	0·01		+	...	...	...	...	...	...	„
	15	0·005		...	...	...	...	...	+	...	14.9.26
	15	0·004		...	... +	...	...	...	...	...	„
	15	0·0033		...	...	... +	...	...	...	...	„
	20	0·0025		...	...	...	...	+	...	...	27.9.26
	20	0·002		...	...	...	...	... +	...	...	„
	17	0·002		...	...	...	...	...	...	S	4.10.26
	15	0·00166		...	...	...	...	...	...	S	„
	15	0·00143		...	...	...	...	...	...	S	„
18	18	1/300 C.S.S. + 0·09	45	...	...	...	...	...	... +	...	9.10.26
	18	„ „ + 0·08	40	...	...	...	...	... +	...	...	„
	15	„ „ + 0·07	35	...	...	...	...	...	... +	...	„
	16	„ „ + 0·06	30	...	...	...	...	...	... +	...	„
	18	„ „ + 0·05	25	...	...	...	...	...	...	S	„
225	15	0·1		...	+	...	...	...	...	...	21.6.26
	18	0·05		...	... +	...	...	...	...	...	„
	18	0·02		...	...	...	+	...	...	...	„
	18	0·0133		...	...	...	... +	...	...	...	„
	20	0·0133		...	...	...	...	... +	...	...	28.6.26
	15	0·01		...	...	...	...	...	...	... +	„
	15	0·0066		...	...	...	...	...	... +	...	„
	15	0·005		...	...	...	...	... +	...	...	22.7.26

TABLE I—*continued.*

Toxin No.	Weight of mouse.	Dose (c.c.)	Number of M.L.Ds. in test dose.	Day of experiment. 1.	2.	3.	4.	5.	6.	7.	Date of test.
225	16	1/300 C.S.S. +0·30	60	...	...	...	+	...	...	...	19.7.26
	15	„ „ +0·25	50	...	...	+ ...	...	...	...	...	„
	20	„ „ +0·20	40	...	...	+	...	...	...	...	„
	20	„ „ +0·20	40	...	...	...	...	...	+ ...	...	17.9.26
	20	„ „ +0·17	35	...	...	...	...	...	+ ...	...	„
	15	„ „ +0·15	30	...	...	...	...	.	...	S	3.8.26
	20	„ „ +0·15	30	...	...	...	...	...	...	...	+ 17.9.26
	18	„ „ +0·125	25	...	...	...	...	+ ...	...	...	„
	15	„ „ +0·125	25	...	...	...	...	...	...	S	27.9.26
	16	„ „ +0·10	20	...	...	...	...	...	...	S	3.8.26
	17	„ „ +0·10	20	...	...	...	...	...	...	S	27.9.26
	16	„ „ +0·075	15	...	...	...	...	...	...	S	„
222	15	1/300 C.S.S. +0·30	...	...	...	...	... +	...	...	...	5.2.26
	16	„ „ +0·29	...	...	+ ...	...	...	...	...	...	„
	16	„ „ +0·28	...	...	...	...	+	...	...	...	„
	16	„ „ +0·27	...	...	...	...	... +	...	...	...	„
	17	„ „ +0·26	...	...	...	...	...	... +	...	...	„
	15	„ „ +0·25	...	...	...	...	...	...	...	Ill, S.	„
	15	„ „ +0·24	...	...	...	...	...	...	...	S	„

THE RELATIVE SENSITIVENESS OF THE RABBIT AND MOUSE TO DYSENTERY TOXIN.

The figures expressing the rabbit M.L.D./mouse M.L.D. ratio will be found in Table II. Similar figures given by Hirszfeld and Przesmycki (1924) for 5 broth toxins are: 10, 9, 4, 3 and 2. It can be calculated from the data given in Table II that the average toxicity of the 17 broth toxins for mice is equal to 190 M.L.Ds. per c.c., whereas the average toxicity of 16 of these toxins for rabbits is 53 M.L.Ds. per c.c.; hence the average rabbit M.L.D./mouse M.L.D. ratio is 3·6. When we take into consideration the relative weights of the rabbits and mice under test, namely 1200 and 17·5 gm., it appears that 1/63,600th c.c. is the M.L.D. for 1 gm. of rabbit, and 1/3325th c.c. is the lethal dose for 1 gm. of mouse, so that the tissues of the rabbit are 20 times more sensitive to the lethal action of dysentery toxin than those of the mouse. This result is confirmed by the work of others, for example, by Zangger (1923), who concludes that, estimated per gramme of body-weight, the mouse is 10 to 30 times less susceptible than the rabbit, although there is no absolute constancy in the relation between the toxicity for the mouse and rabbit. Hirszfeld and Przesmycki (1924) found that, taking into account body-weight, rabbits of an average weight of 1500 gm. were 20 times more susceptible to dysentery toxin than mice of 15 gm.

THE EXISTENCE OF TOXOIDS IN DYSENTERY TOXIN.

Reference to Table II shows that for 17 broth toxins examined the number of M.L.Ds. in the L_+ dose varied from 15 to 70. Toxin 225, which represents a mixture of all the strains, contained 30 M.L.Ds. in the L_+ dose; toxin 222, a three weeks broth filtrate, had 34 M.L.Ds. in the L_+ dose; toxin 203, a

TABLE II.—*Summarizing the Results of Testing a Series of Broth Toxins prepared from Different Strains of the Dysentery Bacillus (Shiga) and grown for 5 weeks at 37° C. in the same batch of Broth The dates are arranged in order of the number of M.L.Ds. in the L_+ dose of toxin as determined by the intravenous method of testing in mice.*

Identification No. of toxin.	Source.	Tests on mice.						M.L.D. for rabbit (c.c.).	Rabbit M.L.D. / Mouse M.L.D. ratio.
		L_+ dose (c.c.).	No. of M.L.Ds. in L_+ dose.	L_0 dose (c.c.).	No. of M.L.Ds. in L_0 dose.	No. of M.L.Ds. in "$L_+ - L_0$."	M.L.D. (c.c.).		
4	National Collection of Type Cultures	0·15	15	0·125	12½	2½	0·01	? 0·1	? 10
13	K 624	<0 10	<25	...	...	...	0·004	0·02	5
15	Lister Institute No. 7	0·25	25	0·20	20	5	0 01	0·04	4
16	Pasteur Institute	0·20	25	0·16	20	5	0·008	0·04	5
1	Lister Institute, Chelsea	0·50	25	0·40	20	5	0·02	0·10	5
17	Dr. O'Brien	0·50	25	0·40	20	5	0·02	0·50	25
12	King George H.P.	0·075	30	0·05	20	10	0·0025	0·01	4
9	National Collection of Type Cultures	0·30	30	0·25	25	5	0·01	0·02	2
7	" " " "	0·15	30	0·10	20	10	0·005	0·02	4
18	Dr. Madsen	0·06	30	0 05	25	5	0·002	0·04	20
3	Bombay	0·28	35	0 24	30	5	0·008	0·04	5
10	550 "Rough"	0·20	40	0·17	35	5	0·005	0·02	4
14	Lister Institute 359	0·132	40	0·10	30	10	0·0033	0·0066	2
11	550 "Smooth"	0·25	50	0·20	40	10	0·005	0·01	2
6	National Collection of Type Cultures	0·35	70	0·25	50	20	0·005	0·0133	2·7
5	" " " "	...	...	...	...	...	0·0033	0 01	3
8	" " " "	...	...	...	...	...	0·04	0·1	2·5
225	Mixture of above toxins	0·15	30	0·10	20	10	0·005	0·0133	2·7
	Comparative Data for Four other Dysentery Toxins.								
222	3 weeks' filtrate—K 624	0·27 (mgm.)	34	0·24	30	4	0·008 (mgm.)	0·025	3
203	Lister Institute sulphated dry toxin	2·5	8	...	...	...	0·3	...	...
Dry toxin	Copenhagen Standard	0·33	10	...	...	...	0·033	...	...
" "	Pasteur Institute Strain 30	0·4	16	...	...	...	0·025	...	...

dry sulphated toxin, had 8 M.L.Ds. only in the L_+ dose; there were 10 M.L.Ds. in the L_+ dose of the Copenhagen dry standard toxin, and 16 M.L.Ds. in the L_+ dose of the Pasteur Institute dry toxin. It is interesting to find that all the broth toxins have a larger number of M.L.Ds. in the L_+ dose than the dry bacillary toxins, and it is natural to think that the reason lies in the mode of preparation of these toxins, which is likely to bring about a considerable degradation of the acute toxin. The Copenhagen dry toxin underwent a preliminary heating to 58° C. before drying, and this circumstance probably explains the smaller number of M.L.Ds. in the L_+ dose as compared with the Pasteur Institute dry toxin. The results, taken as a whole, make it clear that in dysentery toxin there is, besides the acute poison, an atoxic constituent which can bind antitoxin, and which therefore corresponds with Ehrlich's definition of toxoid. The figures in the table representing the number of M.L.Ds. in the L_o dose, and in the "$L_+ - L_o$" value, are of less certain significance because they depend on the arbitrary criterion we have adopted in defining the L_o dose, and moreover the measurements of the test doses were not subdivided enough to justify more than a rough comparison; however, they are inserted for the sake of completeness. The determination of an exact L_o value by the method of intravenous injection into mice would presumably depend on the absence of symptoms of illness, a criterion, where the mouse is the subject of experiment, upon which it is very difficult to form a judgment. Ehrlich did not find it easy to estimate the L_o limit of diphtheria toxin, although in addition to general symptoms of illness he was able to observe inflammatory signs at the site of inoculation. Since compiling this table, Ehrlich's paper (1897) on the standardization of anti-diphtheria serum, which contains his observations on the constants of a number of toxins, has been consulted, and the data have been arranged in a manner similar to those of Table II (Table III). The range of variation of the M.L.Ds. in the L_+ and L_o values agrees in a striking way, but we do not know whether or not the resemblance is significant.

Additional proof that dysentery toxin does not consist of a simple unalterable antigen is afforded by the fact that toxins rendered atoxic by chemical means can give rise to dysentery antitoxin, as the following example shows:

The bulk of the mixed toluolized broth cultures already referred to was filtered through a Seitz filter, 0·6% formalin was added, and the toxin was then kept at 37° C. for 4 weeks. This formolized toxin was used for the immunization of a fresh horse, "May." The M.L.D. of the untreated mixed toxin as tested intravenously on rabbits was 1/75th c.c., whereas after the formalin treatment 5 c.c. given intravenously to a young rabbit proved to be harmless. The horse received successive doses of 10, 50, 100 and 200 c.c. of the toxin. Nineteen days after the last dose a sample of the serum contained 200 provisional Copenhagen antitoxin units per c.c. A subsequent course of 50, 100 and 200 c.c. was given, and 7 days after the last dose a sample from a bleeding was found to have a titre of 300 antitoxin units per c.c. as estimated by the neutralizing dose of the serum. Although, then, the formolized product was at least 375 times weaker than the original toxin, it was yet capable of acting as an efficient antigen for the production of antitoxin. Dumas, Ramon and Bilal (1925) have reported similar results.

TABLE III.—*In which the Values for* 12 *Diphtheria Toxins, worked out by Ehrlich* (1897), *are arranged in a manner similar to that in Table II.*

No. of toxin.	L+ dose (c.c.).	No. of M.L.Ds. in L+ dose.	L_o dose (c.c.).	No. of M.L.Ds. in L_o dose.	No. of M.L.Ds. in "L+—L_o" (D.)	M.L.D. (c.c.).	Remarks.
10	0·0292	29·2	0·0275	27·5	1·7	0·001	Fluid concentrated toxin (a solution of a precipitated toxin).
3	0·48	39	0·415	33·2	5·8	0·0125	Toluol toxin.
4	0·355	39·4	0·305	33·4	6	0·009	„ „
1	2·8	40	2·3	33	7	0·07	„ „
2	1·25	42	0·95	32	10	0·03	„ „
8	0·59	42	0·5	35·7	6·3	0·014	Contained 0·5% carbolic acid.
5	1·15	57·5	0·95	47·5	10	0·02	Toluol toxin.
7	1·26	76·3	0·9	54·4	22	0·0165	Contained 0·2% trikresol.
12	0·25	100	0·125	50	50	0·0025	Tested immediately after harvesting.
11	0·0084	112	0·0063	84	28	(gr.) 0·000075 (c.c.)	Dry concentrated toxin.
6	3·05	113	2·6	96	17	0·027	Toluol toxin.
9	0·48	123	0·42	108	15	0·0039	Antinosin—glycerine toxin.

NOTE.—For the diphtheria toxins analysed above the number of M.L.Ds. in the L_+ dose varies from 29·2 : 123 = 1 : 4·2.

For the dysentery toxins analysed in Table II the number of M.L.Ds. in the L_+ dose varies from 15 : 70 = 1 : 4·7.

Similarly for the diphtheria toxins the M.L.Ds. in the L_o dose varies from 27·5 : 108 = 1 : 3·9.

For the dysentery toxins the M.L.Ds. in the L_o dose varies from 12·5 : 50 = 1 : 4.

Variation of "D" ("L_+—L_o") value for the diphtheria toxins is 1·7 : 28 = 1 : 17.

Variation of "D" ("L_+—L_o") value for the dysentery toxins is 2·5 : 20 = 1 : 8.

Variation of M.L.D. for the diphtheria toxins = 0·0039 : 0·07 = 1 : 18.

Variation of M.L.D. for the dysentery toxins = 0·002 : 0·02 = 1 : 10.

Doerr (1924) expressed the opinion that owing to the varying susceptibility of the mouse the exact ascertainment of the composition of various toxic solutions, and especially the proof of atoxic binding constituents of the toxin, must be rendered very difficult. Fukuhara (1919), Hirszfeld and Przesmycki (1924), and Shiga and his colleagues (1924), take the view that the toxin molecules lose their toxicity without the formation of toxoid, and that the binding property of dysentery toxin by antitoxin is reduced in proportion to the decreasing toxicity of the toxin. On the other hand, in the resolutions adopted by the Conference in 1924, the fact is stated without comment that when the same multiple of a certain lethal dose of different toxins is used in the titration of a serum different results are sometimes obtained. A good example is provided in the paper by Madsen to the Conference in 1924, which contains Jensen's results. Jensen used two test toxins: (1) a dry bacillary toxin, and (2) a broth toxin (three weeks old filtrate). Test doses of each of these containing 10 certain lethal doses were titrated against 5 sera (German, English, Polish, Japanese and Lister Institute serum). The amount of each of these sera required to give an L_+ value when the dry toxin was used was twice the corresponding amount of serum required when pitted against the broth toxin.

This is an interesting result, and agrees with the conclusion stated above that the 17 broth toxins investigated all contained relatively less toxoid than the dry standard Copenhagen toxin. Finally, Schlossberger and Hartoch (1924) showed that with dysentery-toxin solutions the antitoxin-neutralizing property runs in no way parallel with their toxicity. They prepared 4 toxins, (1) a dry bacillary toxin, (2) "Waschwasser" toxin, (3) whole toxin, and (4) broth toxin, and found that for neutralization of 5 M.L.Ds. of these toxins quite variable amounts of antitoxin were required—up to a threefold difference—a result which, they considered, contradicts the statements of Fukuhara and others. They conclude that atoxic but antitoxin-binding substances—so-called toxoid modifications—were present in varying amounts.

THE RELATIVE SUITABILITY OF VARIOUS TOXIN PREPARATIONS FOR USE AS A TEST TOXIN.

Kolle, Schlossberger and Prigge (1924) compared the antigenic properties of different kinds of toxin by making monovalent sera in rabbits. They used broth toxins, "washwater" toxins, and a dry bacillary toxin, and found that the antitoxic sera prepared by them exerted an identical action towards the various toxins, and they therefore concluded that such toxins can be used indifferently for titrating anti-dysentery sera. Doerr (1924) co-operated with other members of the Conference in titrating sera, and concluded that the agreement was quite satisfactory considering that different test toxins were used, including a broth toxin and a dry bacillary toxin. Schlossberger and Hartoch (1924) used the L_+ dose of the 4 toxins already referred to against 3 different standard sera, and found that the antitoxin values agreed satisfactorily. Tarassevitch and his colleagues (1924) also came to the conclusion that the titration of serum can be carried out with different toxins—for example, filtrates of broth cultures, and killed bacteria.

That any toxic preparation of the dysentery bacillus can be used as a test toxin for titrating sera admits of little doubt, but the question whether one kind is to be preferred to another remains an open one. The Conference of 1924 recommended the use of a dry toxin in order to obtain uniformity in the tests carried out by the members of the Conference with the newly-prepared standard serum, but there does not seem to be any advantage in choosing a dry toxin for routine test purposes. Indeed this kind of toxin possesses several disadvantages. Shiga (1924) has pointed out that it is somewhat insoluble and consequently troublesome to manipulate. It is certainly difficult to obtain more than a very fine suspension of particles, so that it is natural to wonder whether all the toxin molecules are in a state of solution, or whether some are enclosed in non-specific bacterial *débris*, or adsorbed upon non-specific particles. A dry toxin has been used for a good many years in this laboratory, but it has never been regarded as an ideal test toxin. For example, to avoid agglutination of the suspension by the serum under test, it has been the practice here to inject the dose of serum and toxin immediately after mixing them. As long as there was no standard serum available, a dry toxin, on account of its stability, served a useful purpose, but now we believe that the germ-free broth filtrate is preferable. Zangger (1923), who also thought that

broth toxins were suitable for titration purposes, found no evidence that non-specific reactions due to broth or bacterial constituents were the cause of irregularities in the tests, and, so far as we can judge, our experience has been the same. Ionescu-Mihaesti and Combiescu (1924) noted that the regularity of the tests increased when a liquid toxin was used instead of the killed bacterial bodies.

It is possible that an ideal test toxin will be found to consist of a filtered autolysate of dysentery bacilli prepared in such a way, for example, by the adjustment of the reaction and the buffering action of added salts, that it preserves its character for a long time when kept under the usual conditions of storage, but in the meantime it would seem best to discard the dry bacterial toxin in favour of a broth toxin for ordinary routine testing of sera. The general opinion is that toxins prepared from broth cultures of the dysentery bacillus are decidedly more stable than diphtheria toxin. Toxin No. 222 (see Table II), a filtered broth toxin kept under toluol in the cold room, has remained stable for at least one year. Of 4 broth toxins investigated by Zangger (1923), two kept their toxicity unchanged after one year, and one showed perhaps a slight weakening; in one the toxicity dropped, but this might have been due to accidental contamination. Even if the stability of dysentery toxin were of an order equal to that of diphtheria toxin, there is no reason whatever why it should not be used as a test toxin, other things being equal, so long as it is standardized at appropriate intervals with the aid of a standard serum. The Conference at first arranged that 1/300th c.c. of a 1% glycerine-saline solution of the standard dry serum should be equivalent to 1 unit, *i. e.* the amount of serum which fails to protect a majority of mice against 10 M.L.Ds. of the dry standard toxin. A later decision makes the unit equivalent to 1/200th c.c. of the standard solution of serum, *i. e.* the amount of serum which protects a majority of mice against the test dose of toxin. It would seem preferable for the sake of uniformity to follow as far as possible the principles of testing laid down by Ehrlich for anti-diphtheria serum and to make the unit correspond to a positive result—that is, the death of the test animals within a prescribed period of time, especially since the two values are determinable to the same degree of accuracy. The testing of anti-dysentery sera would thus fall exactly into line with the familiar and simple procedure used in testing anti-diphtheria sera, and the inclusion of a control series of tests with a standard serum would thereby be rendered unnecessary.

SUMMARY OF RESULTS.

(1) Eighteen broth toxins have been investigated and compared with a sulphated toxin and with two dry bacillary toxins.

(2) Tests carried out to determine the M.L.D. of the broth toxins gave more regular results in mice than in rabbits. The rabbit per gramme of body-weight is 20 times more sensitive to the lethal action of dysentery toxin than the mouse. A satisfactory degree of regularity was obtained with toxin-serum mixtures given intravenously to mice. The use of mice derived from a healthy uniform stock is an important factor in achieving regular results.

(3) Dysentery toxins contain toxoid derivatives in varying proportions.

The proportion of toxoids is considerably less in broth toxins than in dry bacillary or sulphated toxins.

(4) For the routine titration of anti-dysentery sera the mouse is to be preferred to the rabbit.

(5) Broth toxins can be used as test toxins for the routine titration of anti-dysentery sera provided that they are occasionally checked with the aid of a standard serum. A control series of tests with a standard serum would seem to be unnecessary.

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THE ACTION OF SULPHONAL ON RABBITS.

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THE object of this research lay in an investigation of the effects of sulphonal upon rabbits, particular attention being paid to the urinary system. It was stated by G. M. Robertson (1911) in a paper on the continuous administration of sulphonal to man that nephritis had been produced by the experimental use of the drug. Unfortunately, no account or reference to the experiments was given.

METHOD OF ADMINISTRATION AND DOSAGE.

Nine rabbits, weighing from 1500–2000 gm., were fed on minced cabbage containing sulphonal. The dose varied from 1 to 3 gm. a day in most cases, with the exception of one rabbit, which ate 7 gm. The dose was that found to induce intoxication, and when severe effects were noted the drug was withheld, the animals being allowed to recover before the administration of the drug was resumed.

Six rabbits received injections of a warm solution of sulphonal into the peritoneal cavity. The solution employed was a 1·25% solution of the drug in sterile water, administered at a temperature between 50°–60°C. By this means doses of from 250 mgm./kilo were given. The lethal dose was found to be in the region of 350 mgm./kilo.

Routine Observations.

Before and during the experiments routine examinations were made of the urine, the blood, and the general condition of the animals. The urine was examined for the presence of albumen, blood and hæmatoporphyrin. Estimations were also made of the urinary urea, the creatinine and the chloride content of the urine. A note was made of the total urinary output in each 24 hours. The creatinine and the chloride estimations were suspended after a time, when the results obtained were found to be in accord with those obtained from the urine of healthy animals. The blood-urea content was estimated by the method of MacLean, before experiment, during experiment and also upon the heart blood of certain rabbits at death (Rabbits S7, S8 and S9). The animals were examined from day to day for signs such as an unsteadiness of gait, indicative of sulphonal intoxication, wasting, loss of appetite, and the occurrence of diarrhœa.

One hundred healthy animals, tested before experiment, formed a series of controls. The criterion of health was taken to be a good appetite, a fairly

constant weight, the absence of diarrhœa and a satisfactory renal function. To these were added the findings at autopsy and the results of a histological examination of the organs. The routine followed has been described by Dunn, Haworth and Jones (1924), and was used by Dunn and Polson (1926).

The protocols of these experiments are shown in Tables I, II, III.

Effects on the Behaviour of the Animals.

About two hours after the ingestion of sulphonal the animals became increasingly stupid, losing interest in their surroundings and becoming neglectful of their food. As the influence of the drug increased, a weakness of the hind limbs and an ataxia developed, so that if an animal were made to turn quickly, it tended to fall to one side. This ataxia increased to such an extent that the least change in its posture was accompanied by a loss of balance. Later a complete flaccid paresis of the hind limbs followed. During this time the animals still made feeble attempts to take food, but were impeded by a jerky movement of the head and jaws. With still larger doses the paresis was followed by a prostration in which the animals lay on their sides. Even from this degree of intoxication the animals were able to recover, and it was not then possible to detect any alteration in their behaviour. If the dosage were carried further, the prostration was followed by periodic attacks of clonic convulsions in the limbs, the fore and hind limbs being contracted briskly some thirty times during each attack, while, as the intoxication advanced, the intervals between the attacks gradually shortened, and finally the animals became comatose. They were killed at this stage, when it was thought almost certain that they would have died.

A similar series of events followed the administration of the drug into the peritoneal cavity, but the time of onset of signs was much shorter, they being noticed within half an hour. One animal (S11) developed a flaccid paresis of the hind limbs within ten minutes, so that it was considered the animal had broken its back, but after a time, since it had not in this case been thought necessary to kill at once, the animal recovered. The next day, when the dose was repeated, the same results were produced.

(Preliminary experiments, performed on guinea-pigs, showed that they behaved in the same way as rabbits when under the influence of sulphonal. The lethal dose for these animals was found to be in the region of 0·65 gm.)

Effects on the Urinary System.

Sulphonal appears to disturb the bladder mechanism, causing retention of urine, and this was seen on three separate occasions during the experiment on Rabbit S3. On each occasion the period during which no urine was passed was followed by polyuria. After three days of urinary retention this animal passed 187 c.c. of urine, showing no chemical abnormality to suggest the onset of nephritis. The sample yielded 3·83% of urea and had a urea content of 7·16 gm. On one day alone did the urinary urea fall below the level of 2%, and on that day it was 1·79%. During the remaining thirty-eight days of experiment the urinary urea ranged from 2% to 4·78%. The urea output was in keeping with these figures, and, as has been stated, it reached

TABLE I.—*Protocols of Sulphonal Feeding Experiments.*

Rabbit.	Days under experiment.	Days when sulphonal given.	Days when effects severe.	Total drug received in grammes.	Kidneys.	Bladder.	Days of urinary retention.	Hp.*	Average urinary output during experiment.	Average urinary urea % during experiment.	Average urea output during experiment.	Blood-urea prior to experiment in mgm./100 c.c.	Blood-urea during experiment in mgm./100 c.c.	Blood-urea: Heart blood in mgm./100 c.c.	Stomach.	Brain: Presence of abnormal material.
S 1.	44.	44.	11.	65.	Congested.	Vessels tortuous, empty	0	...	68 c.c.	2·052%	1·166 gm.	20·3	42·5	...	No petechiæ; no erosion	No material present.
S 2.	44.	41.	14.	39.	*Nil*	60 c.c.	1	...	58 c.c.	2·482%	1·330 gm.	19·8	43·0	...	*Nil*	Cerebral cortex, cerebellum, mid-brain and medulla.
S 3.	39.	29.	14.	25.	Dilatation of collecting tubules	101 c.c.	7	++	41 c.c.	2·500%	1·233 gm.	14·2	42·5	...	Erosion at fundus	Medulla.
S 5.	61.	34.	18.	28.	*Nil*	5 c.c.	1	...	41 c.c.	3·832%	1·088 gm.	...	33·0	...	Erosion at pylorus; petechiæ at fundus	None.
S 6.	11.	9.	4.	13.	*Nil*	74 c.c.	0	...	47 c.c.	2·060%	1·000 gm.	28·2	...	...	Petechiæ at fundus	„
S 7.	34.	24.	10.	29.	*Nil*	30 c.c.	0	+++	29 c.c.	3·190%	0·944 gm.	26·0	...	79	Ditto	„
S 8.	100.	52.	18.	70.	Congested.	33 c.c.	3	+	30 c.c. Average of seventy readings	3·660%	0·905 gm.	...	39·0 48·5	89·6	Erosion and petechiæ at fundus	Cerebellum and medulla.
S 9.	46.	16.	10.	15.	*Nil*	93 c.c.	3 prior to experiment; 1 during experiment	+	29 c.c. Average of ten readings	4·290%	1·349 gm.	...	...	89·5	Erosion and petechiæ at fundus	Mid-brain and medulla.
S 4.	4.	4.	0.	2.	Back broken and animal killed											

* Hp. = Hæmatoporphyrinuria.

TABLE II.—*Protocols of the Healthy Control Rabbits.*

Rabbit.	Days of observation.	Average weight.	Food.	Average water intake.	Average urine output.	Average urea %.	Average total urea.	Urea concentration factor.	Blood-urea: Dry diet, mgm./100 c.c.	Brain: Abnormal material.	Notes on fixation of brain, etc.
N 360	9	2150 gm.	All taken	25 c.c.	62 c.c.	2·407%	1·334 gm.	132	32	Present	Brain fixed in alcohol.
N 364	20	1480 gm.	Nearly all taken	60 c.c.	86 c.c.	2·110%	1·858 gm.	60·4	32·1	Absent	„ „ „
N 365	10	2660 gm.	All taken	110 c.c.	95 c.c.	3·759%	3·255 gm.	154	26·7	Present	„ „ „
N 376	6	1670 gm.	„	140 c.c.	39 c.c.	3·173%	0·817 gm.	158	20·4	Absent	„ „ „ Old rabbit (2 years in cages).
N 379	5	2050 gm.	„	65 c.c.	19 c.c.	1·495%	0·352 gm.	43·7	19	Present	Brain fixed in alcohol.
N 380	6	1590 gm.	„	80 c.c.	35 c.c.	4·602%	0·869 gm.	643	30·5	„	„ „ „
N 381	6	1810 gm.	„	125 c.c.	35 c.c.	2·390%	0·735 gm.	195	22·4	Absent	„ „ „
					Bladder urine.						
N 382	6	1560 gm.	Nearly all taken	40 c.c.	None on 4 days; 2 c.c. in bladder	4·650%	0·093 gm.	114·5	40·6	Present	„ „ „
N 384	6	1660 gm.	All taken	50 c.c.	37 c.c.	3·346%	1·392 gm.	140	32·4	Absent	„ „ „
N 385	6	1930 gm.	„	100 c.c.	74 c.c.	2·823%	1·248 gm.	32·3	45·6	„	„ „ „
S 24	10	1380 gm.	„	25 c.c.	20 c.c.	3·538%	0·634 gm.	221	15·9	„	Half in corrosive. „ 10% formol saline.
S 25	10	1540 gm.	Nearly all taken	35 c.c.	25 c.c.	2·870%	0·594 gm.	284	23·8	Present	„ 96% ethyl alcohol. „ 10% formol saline.
S 26	10	2170 gm.	All taken	25 c.c.	38 c.c.	3·938%	1·510 gm.	118	35	„	„ Zenker's fluid. „ 96% ethyl alcohol.
S 27	10	1500 gm.	„	75 c.c.	34 c.c.	3·160%	0·676 gm.	574	7·8	Absent	„ formol Muller. „ 10% formol saline.

TABLE III.—*Protocols—Intra-Peritoneal Administration of Sulphonal.*

Rabbit.	Days under experiment.	Days when dose given.	Days when effects severe.	Dose.	Total drug received.	Kidneys.	Average urinary output during experiment.	Average urinary urea % during experiment.	Average urea output during experiment.	Peritoneum.	Blood-urea during experiment.	Brain deposit.
S 10	2	2	2	370 mgm. per kilo	0·75 gm.	*Nil*	33 c.c.	3·550%	1·140 gm.	*Nil*	...	None.
S 11	2	2	2	320 mgm. per kilo	1·0 gm.	„	54 c.c.	2·029%	0·471 gm.	„	...	Not examined.
S 12	23	16	12	330 mgm. per kilo	7·0 gm.	„	77 c.c.	2·119%	1·511 gm.	Some local peritonitis	24·0	Ditto.
S 13	6	5	4	350 mgm. per kilo	1·75 gm.	„	60 c.c.	2·200%	1·374 gm.	Ditto	...	None.
S 15	20	14	12	250 mgm. per kilo	6·50 gm.	„	68 c.c.	4·385%	1·458 gm.	„	24·5	Not examined.
S 16	38	23	14	250 mgm. per kilo	8·75 gm.	„	50 c.c.	3·178%	1·460 gm.	„	13·4	Ditto.

the high level of 7·16 gm. These findings, together with a consideration of the chart of daily observations (Chart 1), showed that the disturbance was due to retention of urine rather than an alteration of secretion.

The remainder of the animals suffered no changes in the urine or in the blood suggesting the onset of nephritis. The observations were in accord with those made on healthy animals. A chart illustrating these results is given (Chart 2).

No blood was found in the specimens of urine examined and no definite evidence of albuminuria was obtained. Hæmatoporphyrin was found in the urine of four rabbits (S3, S7, S8 and S9). Although the pigment was found in the urine for several days, the animals were able to recover before succumbing later to further doses of sulphonal.

There was, therefore, no evidence of a chemical nature to show that nephritis had been produced by the administration of sulphonal. Nor was any apparent change in the renal function to be associated with the occurrence of hæmatoporphyrinuria.

The Kidneys.

Naked-eye examination.—The kidneys showed no lesion visible to the naked eye. Swelling, œdema and the presence of subcapsular hæmorrhages were not observed.

Histological examination.—The kidney of Rabbit S3, an animal which suffered from retention of urine, was the only one in which any abnormality was found. Here the collecting tubules were dilated. The convoluted tubules and the glomeruli were apparently healthy. It is to be noted that this was the animal in which the hæmatoporphyrinuria was severe.

Thus the histological and the naked-eye evidence supported the chemical findings, in that no evidence of nephritis was found to be associated with sulphonal poisoning and hæmatoporphyrinuria. The dilation of the collecting tubules was regarded as compatible with retention of urine and not as a sign of nephritis.

Effects on the Digestive System.

Of the nine rabbits fed on sulphonal, six presented naked-eye lesions in the stomach, in the form of petechial hæmorrhages and gastric erosions. The former have been described by Stokvis (1895) occurring in the stomachs of dogs and rabbits fed on sulphonal. The petechial hæmorrhages were seen alone or together with an erosion. Not more than one erosion was found in each stomach. This lesion was found to affect the fundus of the stomach of three rabbits (S3, S8 and S9), while that in the stomach of Rabbit S5 was found near the pylorus. The petechial hæmorrhages affected the fundus, but in the case of Rabbit S6 there were many hæmorrhages scattered over the stomach. Microscopical examination showed that the petechial hæmorrhages and the erosions involved only the superficial layer of the gastric mucosa. The surrounding mucosa was little damaged and there was but slight evidence of inflammatory reaction. No blood-cells were seen in the hæmorrhages, the brown spots consisting of amorphous blood-pigment. No erosions or petechial hæmorrhages were found in the stomachs of those animals receiving sulphonal into the peritoneum. These lesions are considered to be due to a local toxic

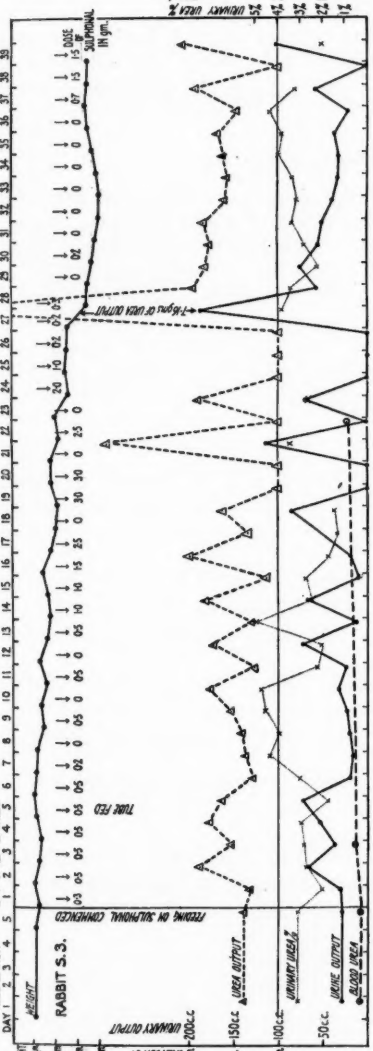

CHART 1.

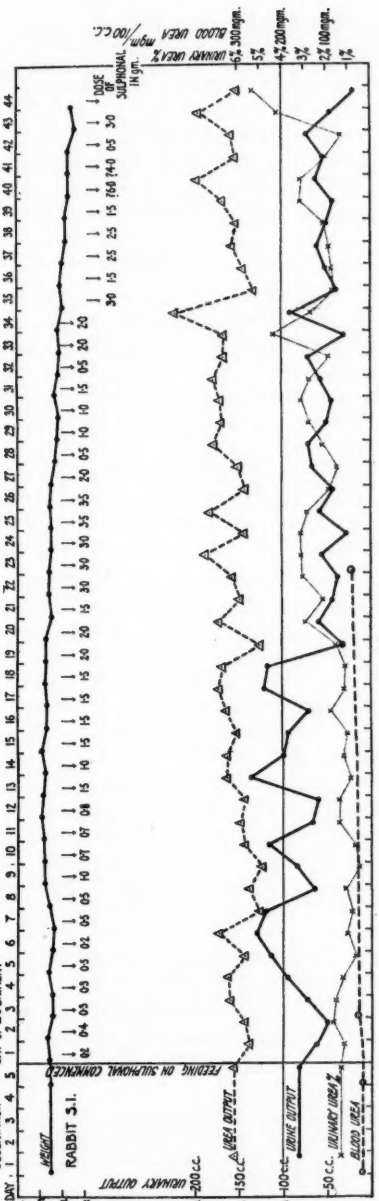

CHART 2.

action of the drug, and the erosions are thought to be produced also by the contact of the sharp and relatively insoluble crystals of the drug with the gastric mucosa.

Effects on the Liver.

The liver of Rabbit S6, an animal suffering from acute intoxication, was loaded with fat, only the periphery of the lobules being free. In all, nine of the livers were examined, and of these there was but one showing severe fatty change. In two no fat was detected.

Effects on the Central Nervous System.

Very scanty evidence of change was found. A certain abnormal material was found, giving a positive reaction with the specific stains for mucin, but an examination of the brains of fourteen healthy control animals showed it to be present in half of each of the groups of brains. This will form the subject of a subsequent paper. In October, 1926, Mott, Woodhouse and Pickworth published a paper on the action of hypnotic drugs on the nervous system of cats and monkeys. In this they describe a "mucinoid deposit" which was produced in the brains of their experimental animals, and which seems to correspond to that found in this series of experiments.

CONCLUSIONS.

1. That rabbits weighing from 1500 to 2000 gm. required from 1 to 3 gm. of sulphonal to induce profound effects, when the drug was administered by the alimentary tract. If the drug were given into the peritoneum, a dose of 250 mgm./kilo was sufficient.
2. That there was no evidence to show that nephritis was produced by sulphonal intoxication, nor was this lesion associated with hæmatoporphyrinuria.
3. That there is evidence to suggest that sulphonal may cause retention of urine.
4. That hæmatoporphyrinuria occurred after large doses of sulphonal, but the animals were able to recover, if the drug were immediately withheld.
5. That sulphonal produced gastric erosions and petechial hæmorrhages when administered by the alimentary tract.
6. That fatty change in the liver was the exception rather than the rule.

I wish to express my thanks to Prof. Shaw Dunn, Dr. C. C. Easterbrook and to Dr. J. B. Duguid for the interest they have taken in this work, and to thank the Government Grant Committee of the Royal Society for a grant towards the expenses of this research.

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AN ABNORMAL MATERIAL OCCURRING IN THE BRAINS OF RABBITS.

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MOTT, Pickworth and Woodhouse (1926), in a paper dealing with the action of hypnotic drugs upon the central nervous system of cats and monkeys, described a "mucinoid" deposit occurring in the brains of their experimental animals. They believe that this deposit was produced by the action of the drugs, of which sulphonal was one, on the nervous system. The writer had treated several rabbits with sulphonal, and, subsequent to the publication of Mott's work, the brains of these animals and those of several healthy animals, were examined for the presence of this "mucinoid" deposit. A substance, bearing a close resemblance to that described by Mott and his colleagues, was found with equal frequency in the brains of the experimental animals and those of the healthy animals.

TECHNIQUE.

The brains of fourteen apparently healthy animals and eight experimental animals were examined. The control animals were under observation from 5 to 20 days, and were found to have a good appetite, a fairly constant weight, no diarrhœa, and a satisfactory renal function. Histological examination of their organs, other than the brain, revealed no pathological lesion. The experimental animals received daily doses of sulphonal in their food, the dose being one such as was found to induce intoxication. The total amount of the drug received by these animals ranged from 13 to 70 gm., while the experiments lasted from 11 to 100 days.

The brains were removed within half-an-hour of the death of the animal. They were cut in slices about 4 mm. thick, and fixed in 96% ethyl alcohol for 24 hours. The slices were then embedded in paraffin, all the brains being treated in the same manner. Sections 6μ thick were cut from each slice and mounted in series. One series was stained by the method of Nissl, the original Nissl stain or 2% toluidin blue being used, and a second was stained with hæmalum and eosin.

The protocols concerning these animals will be found in a previous publication (Polson, 1927).

FINDINGS IN THE CENTRAL NERVOUS SYSTEM.

In seven of the control brains, and in four of those from the experimental animals, the presence of an abnormal material, staining metachromatically with toluidin blue and Nissl's stain, was noted. This material presented three

different appearances, and sometimes all three were seen in the same section of brain. In the first place, the material appeared as uneven rings, varying from 5 μ–40 μ in diameter, scattered over the white matter (Figs. 1 and 2); secondly, as a finely divided deposit, having the same distribution as the rings, and seen with equal frequency (Fig. 3); and thirdly and less frequently as homogeneous globules (Figs. 4 and 11).

The red uneven rings seen in the sections stained with toluidin blue presented the most striking appearance of the material. The central area of the rings was unstained or but faintly stained, and it was possible to see the structure of the enclosed nervous tissue. In the second and third cases the particles were stained a deep red colour.

When the sections stained with toluidin blue were examined by light passed through a "Wratten G" filter, the material appeared a deep red on a green-blue background, as did that described by Mott. With hæmalum and eosin it was stained blue, and the rings now appeared as discs with a dark periphery (Figs. 5, 6 and 7). In the unstained sections the material could be recognized as translucent masses with a refractile periphery. The rings and the red masses were found to be composed of amorphous granules, lying in the spaces between the nerve-fibres, and the latter were frequently seen to be fragmented in the affected areas. Nerve-cells in the mid-brain, pons and medulla were seen to contain red particles (Fig. 3).

The material was present always in the white matter of the cerebrum and the cerebellum (Figs. 5 and 6). It occurred frequently in the lateral and dorsal regions of the mid-brain, pons, and medulla, but at no time was it found in the anterior region. In several sections the material was also seen to be deposited in the white matter bounding the perivascular spaces (Figs. 4 and 8), while it was seen less frequently in the perivascular spaces themselves (Figs. 9 and 10). It was occasionally found in the subarachnoid space (Fig. 11). None of the material was found in the grey matter of the cerebrum or the cerebellum.

The following tests, described by Mott and his colleagues, were performed upon sections from a slice of the medulla of Rabbit S 9, found to contain the material:

(*a*) The application of warm water and saline.
(*b*) The application of strong formol.
(*c*) The application of weak acid and alkali.
(*d*) The application of the following solvents: water, formol, saline, ether, chloroform, xylol, benzol and petroleum ether.
(*e*) The application of Lugol's iodine followed by sulphuric acid.
(*f*) The histo-chemical tests for iron, amyloid and reducing substances.
(*g*) The specific stains for amyloid: methyl violet, congo red and iodine.
(*h*) The specific stains for mucin: mucihæmatin, polychrome methylene blue, toluidin blue and Bismarck brown.

The material appeared to swell slightly with warm water and saline, and was collapsed by strong formol. It gave a positive reaction with the stains for mucin. It was not dissolved by the solvents applied, and remained unaffected by acid and alkali. The histo-chemical tests were negative, and

no reaction was obtained with stains for amyloid. These results are in agreement with those of Mott and his colleagues.

Further experiments included the fixation of brains of healthy rabbits by different fixatives. The material was found to be present in brains fixed by 96% ethyl alcohol, 10% formol saline, a saturated aqueous solution of mercuric chloride and Zenker's fluid. In each case the material presented the same appearances, and was found in one or other of the customary sites, as described above. It was considered that the change might be due to the action of a lipoid solvent on nervous tissue, and half a brain was soaked in chloroform for an hour before fixation. The other half was placed in Ringer's solution for an hour and then fixed. The experiment was inconclusive, since both halves contained an equal amount of the material. A second experiment to test the direct action of sulphonal consisted of the immersion of half a brain in a saturated aqueous solution of the drug for an hour at 37° C. before fixation. A control was made as before. Again both halves contained an equal amount of the material. In a third experiment a 10% solution of sulphonal in chloroform was injected, *viâ* the aorta, into the carotid arteries of a rabbit, the injection lasting twenty minutes. None of the material was found in the brain of this animal.

The Nerve-cells.

As has been stated, finely divided particles of red material were seen in nerve-cells of the mid-brain, pons and medulla. In spite of this, the affected nerve-cells showed none of the characteristic signs of degeneration (Figs. 3 and 4). Some of the nerve-cells, free from the material, tended to stain diffusely with toluidin blue and Nissl's stain, but apart from this no other evidence of neuron degeneration was found.

DISCUSSION.

The material appeared with equal frequency in the brains of the control and the sulphonal-treated animals. In both groups it presented the same appearances and occupied the same sites, so that one may not conclude that sulphonal played any part in its production. Neither does it seem that it is an idiopathic change which can be accentuated by sulphonal, for there was rather more of the material in the control brains than in those of the experimental animals. The possibility that it is an artefact has been considered, but there is much evidence against this explanation. Were the embedding process responsible, then it might be expected that the material would have been found in all the brains, since they were subjected to a standard process. Although four different fixatives were used, the material presented the same features with each. More than ten different stains demonstrated it in the corresponding areas of different sections of the same slice of brain, and any difference in appearance was due to a difference in the intensity of the staining reaction. Although heat was applied to the brains during the embedding process and to the sections stained by Nissl's method, it seems improbable that it played any part in the production of the material, for only half of the series of brains was affected.

It may be concluded, therefore, that this material is a pathological lesion of laboratory animals, unconnected with any experimental procedure. Its origin remains to be determined, but the frequent relationship of the material to the nerve-fibres of the white matter of the brain must be emphasized. As far as may be judged, this material appears to be identical with that of Mott, although he was unable to find any "mucinoid" deposit in the brains of his healthy control animals. This difference may be explained by the frequency with which "normal" rabbits develop pathological lesions in the nervous system (Bender, 1925).

I wish to express my thanks to Prof. Shaw Dunn and to Dr. J. B. Duguid for their advice. I have to thank the Government Grant Committee of the Royal Society for assistance towards the expenses of this research.

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DESCRIPTION OF THE FIGURES.

Fig. 1.—*Cerebellum.* Control rabbit N 379. × 50. Toluidin blue. The uneven rings are seen in the white matter. The granular layer of the cerebellum appears at the lower right-hand corner.

Fig. 2.—*Cerebellum.* Control rabbit N 379. × 300. Toluidin blue. A high-power photograph of the rings seen in Fig. 1.

Fig. 3.—*Cerebellum* and *medulla.* Control rabbit N 340. × 300. Toluidin blue. The material is present in granules and small masses. Some of it is to be seen in a nerve-cell.

Fig. 4.—*Cerebellum* and *medulla.*—The same section as Fig. 3, but a photograph of a different field. The material is seen as homogeneous globules and granular masses in the white matter surrounding the perivascular space.

Fig. 5.—*Cerebrum.* Control rabbit N 380. × 50. H. and E. The material is present in the form of discs with a dark peripheral ring.

Fig. 6.—*Cerebellum.* Control rabbit N 379. × 50. H. and E. A section from the same slice of brain as Fig. 1. The material is seen as discs in the white matter. The granular layer of the cerebellum is seen in the right lower corner.

Fig. 7.—*Cerebrum.* The same section as Fig. 5. × 150. H. and E. The structure of the nervous tissue can be seen through the pale central area of the discs.

Fig. 8.—*Cerebellum* and *medulla.* Control rabbit N 340. × 125. Toluidin blue. Three vessels are seen. The material is present in the white matter immediately surrounding the perivascular spaces. Fig. 4 is a high-power photograph of the vessel on the right.

Fig. 9.—*Medulla.* Control rabbit S 26. × 100. Toluidin blue. Fixation with Zenker's fluid. The material appears in granular masses. The perivascular space of the vessel in the upper part of the photograph contains some of the material.

Fig. 10.—*Medulla.* The same section as Fig. 9. × 500. Toluidin blue. The material, indicated by the arrow, is represented by the haze in the perivascular space.

Fig. 11.—*Mid-brain.* Control rabbit N 340. × 125. Toluidin blue. Some of the material is present in the form of homogeneous masses lying in the sub-arachnoid space.

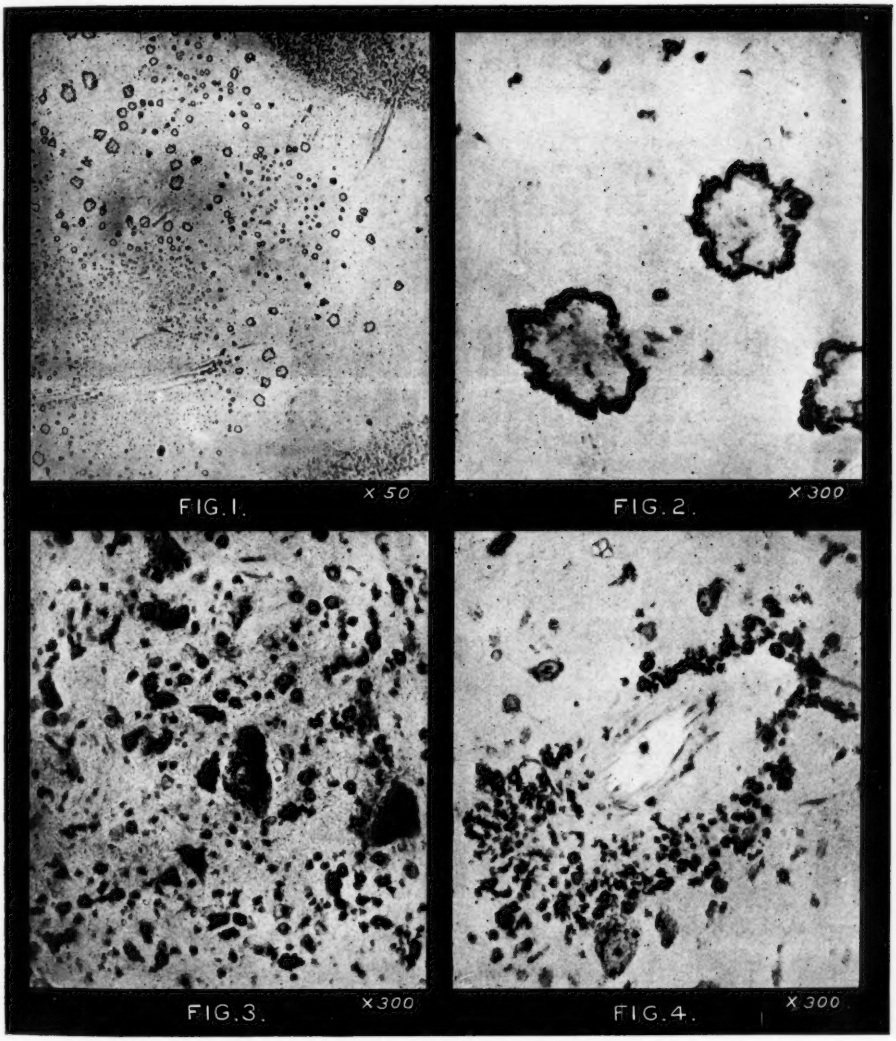

FIG. 1. × 50

FIG. 2. × 300

FIG. 3. × 300

FIG. 4. × 300

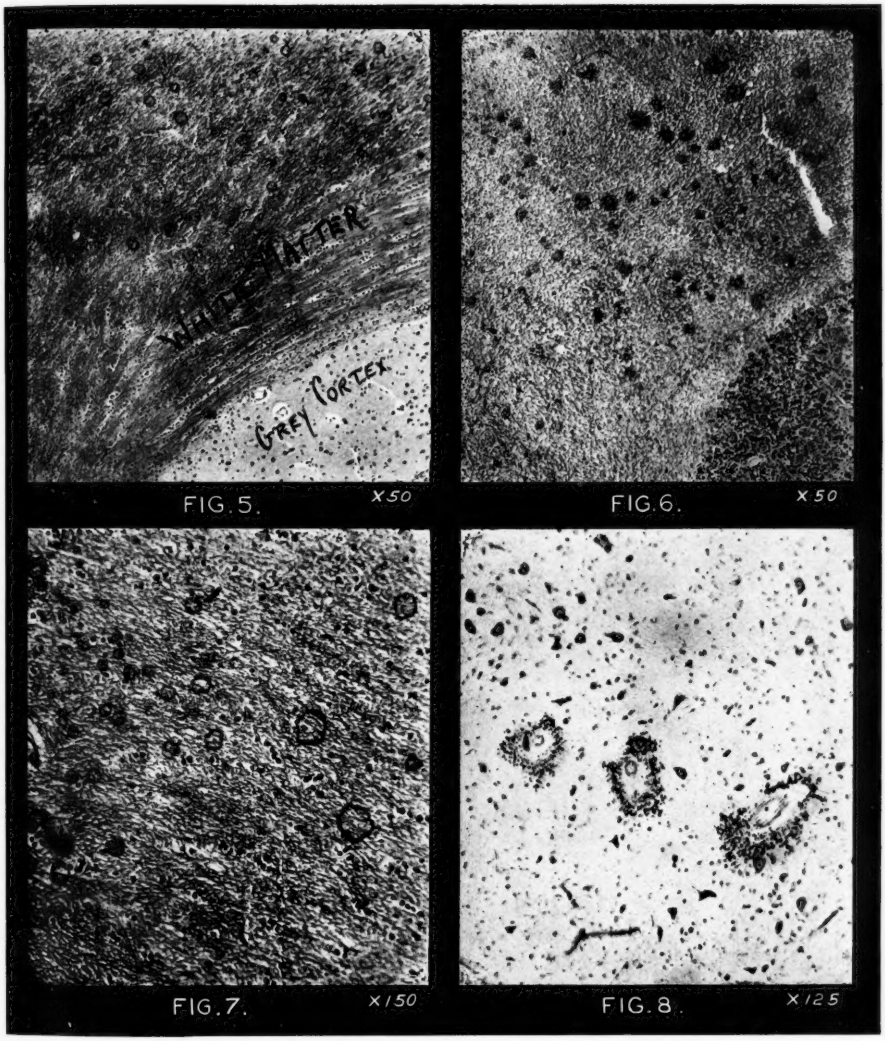

FIG. 5. x 50

FIG. 6. x 50

FIG. 7. x 150

FIG. 8. x 125

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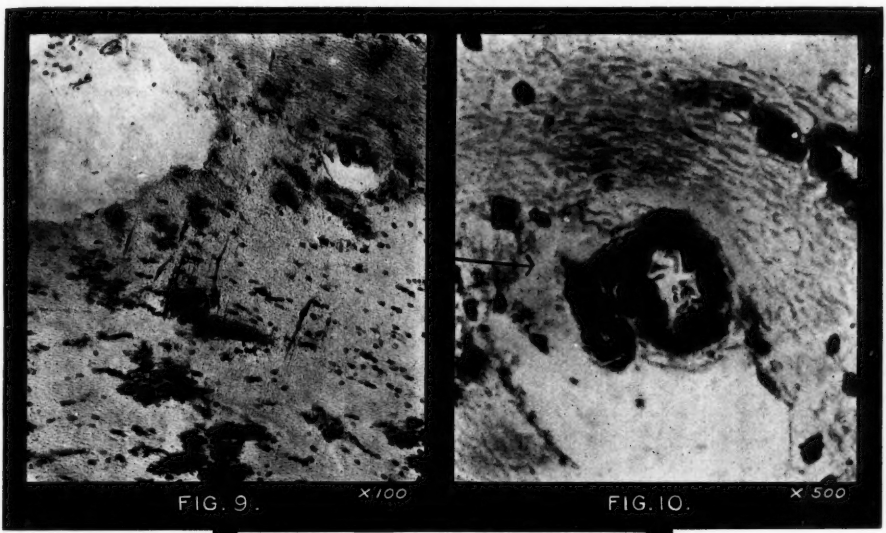

FIG. 9. ×100

FIG. 10. ×500

FIG. 11. ×125

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A QUANTITATIVE METHOD FOR DETERMINING THE FATE OF TUBERCLE BACILLI IMPLANTED IN HUMAN PLASMA.

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Received for publication March 21st, 1927.

THE following study is based on the researches of Wright (1924), who introduced a method for the cultivation of tubercle bacilli in blood or plasma-clots, and for determining the effects exerted by the blood-cells and blood fluids upon these bacilli. He found, among other things, that tubercle bacilli grow more luxuriantly in normal plasma than in that of tuberculous subjects, and also that their growth is checked to a considerable extent by the leucocytes.

In attempting to confirm and extend these important observations, the desirability of a quantitative technique became apparent, and the chief object of this paper is to describe a simple device by which this need may be met. An emulsion of minute carbon particles of known strength is implanted in the blood or plasma along with the tubercle bacilli to serve as a standard with which to compare the number of separate bacilli or colonies. Carbon particles were chosen as being inert and easily recognized microscopically; other bodies, including dead bacilli, starch-granules, precipitated sulphur and lycopodium spores were found less satisfactory.

Certain other technical difficulties met with in the course of the work will also be dealt with.

DETAIL OF THE TECHNIQUE EMPLOYED.

A quantity of *carbo ligni* is powdered, washed, and finally suspended in Ringer's solution, where the larger particles are allowed to settle. The supernatant fluid containing the lighter particles is pipetted off and sterilized by heat; after the suspension has been thoroughly shaken, the number of particles may be enumerated directly in a hæmocytometer chamber, or estimated against red blood-corpuscles in a Romanowsky stained film of a mixture with blood. A suitable concentration is found to be about 20 thousand particles per c.mm.

An emulsion of tubercle bacilli from a recent culture is made with Ringer's solution in a Hayden's bacteriological mortar and placed within a small conical glass tube, which is then sealed and centrifuged for a short time to throw down the larger clumps; the upper layers of the emulsion should then be slightly but distinctly turbid and contain a high percentage of single bacilli.

A mixture of known volumes of the two suspensions—carbon particles and tubercle bacilli—is then prepared, and the ratio between particles and bacilli determined by counts made on a stained film (it is convenient to add also some serum to fix the particles and bacilli to the slide). The bacillary suspension is then adjusted to give a concentration of some 20 to 40 thousand per c.mm., 5% of which with plasma gives a suitable distribution for counting in the clot.

Blood is drawn from a vein in a sterile syringe which has been rinsed with Ringer's solution to obviate hæmolysis. The blood is centrifuged in a paraffined tube surrounded with ice in warm weather to delay clotting. The resulting plasma contains platelets, but is otherwise free from corpuscular elements.

Sterile calibrated pipettes tipped with paraffin are used to place 5 c.mm. of the carbon particle suspension, 5 c.mm. of the bacillary suspension, and 100 c.mm. of plasma on a sterile paraffined slide set on a platform kept cool by a freezing mixture. Here they are thoroughly mixed, and samples of the implanted plasma are drawn into capillary tubes, which are then sealed at both ends; these are placed flat in a warm water-bath so that clotting may occur rapidly before much gravitation of the particles can take place.

Of these tubes one is examined as soon as the clot has formed; another is put aside at room temperature in order to ascertain whether any growth (or lysis) of the bacilli occurs in these circumstances; the rest are incubated in a water-bath at 37°C. and withdrawn for examination after varying intervals. The method of examining the clot is the same in all cases, that is, the tube is cut across and the clot is blown into distilled water, where it is washed free of serum. It is then laid on a slide, allowed to dry, and stained by the Ziehl-Neelsen method. With the help of a square diaphragm in the microscope ocular, the carbon particles and tubercle bacilli are counted field by field across the clot transversely so as to minimize errors due to unequal settling of particles and bacilli before clotting of the plasma.

Enumerations are made firstly in the unincubated clot of the number of single bacilli together with small groups of bacilli—each being regarded as a potential focus of growth. Thus such an initial count might show the presence of 150 foci per 100 carbon particles, the average number of bacilli per focus being therefore 1·2. The group distribution is likewise calculated and expressed in percentages, *e.g.* singles 86, twos 11, and threes 3 per cent. In clots removed after varying periods of incubation similar counts are made, and in this way it is possible to detect any diminution in the number of foci, and any growth as shown by increase in the total number of bacilli and increase in the size of colonies. The actual number of bacilli implanted and grown may be reckoned from their relation to 100 carbon particles whose number per unit volume of plasma is known. The experimental error is believed not to exceed 10%.

RESULTS.

Figs. 1, 2, 3 concern such an experiment made with a certain strain of tubercle bacillus implanted in plasmas of tuberculous and non-tuberculous subjects. It is typical of a number of other experiments made with the same

bacillus. It is readily seen that the total number of bacilli increases much more rapidly in the normal than in the tuberculous plasma. Daily counts

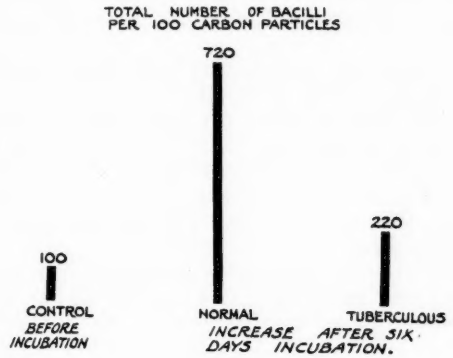

FIG. 1.

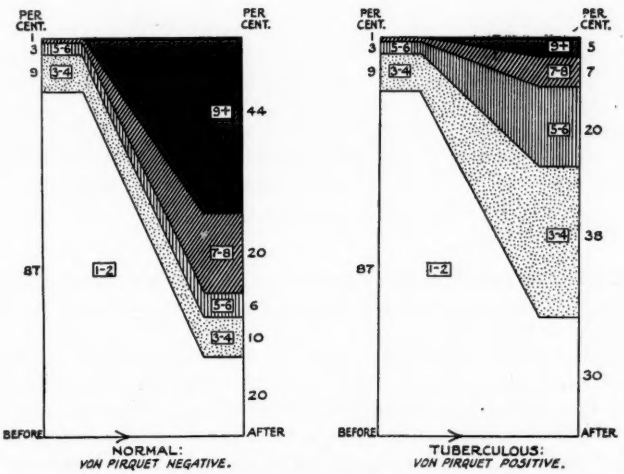

FIG. 2.—Percentage distribution of colonies before and after 6 days' incubation. 1–2 = single bacilli and groups of two. 3–4 = groups of three and four. 5–6 = groups of five and six. 7–8 = groups of seven and eight. 9+ = groups of nine and over.

showed the progressive differentiation: the changes are already evident after 24 hours' incubation, and by the sixth day the size of some colonies has become so great as to preclude accurate counting of their members. In this case the ratio between the number of bacilli in normal as against tuberculous plasma

had become as 720 to 220. The conclusion is further borne out by calculations of the percentage distribution of colonies; it is shown that after the 6-day period, 44% of the total colonies are of the class 9+ in the case of normal plasma, as against 5% in tuberculous plasma. Such observations have confirmed in quantitative fashion the finding of Wright already mentioned.

Control clots left at bench temperature for 6 weeks have shown no growth of bacilli, nor any disappearance of bacilli.

The writer is informed by S. R. Douglas that the bacillus used in these experiments is an avian strain. And since it is known that the avian bacillus differs in serological reactions from human and bovine strains, experiments are now in progress to determine whether normal plasma also favours the growth of these latter strains. It has not yet been possible to reach any generalization on the matter.

With mammalian bacilli there is some evidence of destruction by lysis within the clot, or at all events of a loss of acid-fastness by some bacilli. It

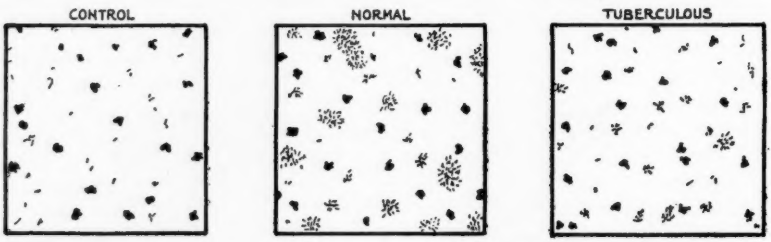

FIG. 3.—Sample microscopic fields.

is possible, however, that unless the emulsion has been made from a recent growth which is the result of repeated subculture, a proportion of the bacilli are not viable to start with.

It was found that there occurred an excessive growth of bacilli at the extremity of the clot as compared with the middle. Relatively consistent counts can be obtained, however, in the central portions of the clot. The growth would thus appear to be related to oxygen supply, for clots in capillary tubes sealed at each end with paraffin to exclude air fail to support growth. Nor do clots washed and incubated in Ringer's solution yield any growth, doubtless because of the removal of the serum in which, as well as in the fibrin meshwork of the clot, the bacilli find nourishment (Wright). But the organisms in clots expressed into and incubated in serum grow well and uniformly.

The practice of implanting citrated or oxalated plasma which is subsequently rendered coagulable by the addition of calcium has been discarded as giving irregular results, but the subject will be returned to on account of the technical facilities such a method would allow.

AUTOLYSIS OF PLASMA-CLOT.

The clot formed by plasma in ordinary glass tubing is liable to undergo a process apparently of lysis, at a speed varying from subject to subject. The phenomenon occurs even at bench temperature, but much more rapidly in the incubator. The rapidity with which this occurs has not been connected with the patient's condition, and occurs indifferently with tuberculous and non-tuberculous plasma, but it was found that it was ascribable essentially to the alkalinity of the glass, which persists to some extent even after washing with distilled water. But when capillary tubes are used which have been drawn from resistance glass, no such liquefaction occurs; this glass is neutral to phenol red and remains so when heated.

The method is being used to study whether there exist differences between patients presenting different clinical types of tuberculosis, or between patients infected with human and bovine bacilli respectively; and, later, it is hoped to determine whether clinical effects obtained by therapeutic measures such as irradiation, etc., can be correlated with changes in the effect of the blood upon the tubercle bacillus.

SUMMARY AND CONCLUSION.

The incorporation of a simple counting device into Wright's method of inculturing tubercle bacilli in human blood or plasma has enabled quantitative experiments to be carried out.

The difficulty which arises from autolysis of plasma-clots on incubation has been overcome by the use of non-alkaline glass.

Working with an avian strain of *B. tuberculosis*, believed to be identical with that employed by A. E. Wright, the finding that tubercle bacilli grow better in normal plasma than in that of tuberculous subjects has been confirmed.

It is a pleasure to record gratitude to Sir Almroth Wright, Dr. L. Colebrook and Capt. S. R. Douglas for advice and criticism. The observations have been made at the Treloar Cripples' Hospital, Alton, and thanks are due to the Medical Superintendent, Sir Henry Gauvain, for according willing access to clinical material under his care. The Medical Research Council has kindly provided a grant, part of which was generously contributed by Sir Horace Plunkett.

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ON THE DEVELOPMENT OF STRAINS OF BACTERIA RESISTANT TO LYSOZYME ACTION AND THE RELATION OF LYSOZYME ACTION TO INTRACELLULAR DIGESTION.

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In a previous communication (Fleming and Allison, 1922) it has been shown that when tears, sputum, egg-white or other lysozyme-containing substance is embedded in an agar plate and the surface of the plate is thickly planted with a microbe specially susceptible to lysozyme action (*e. g. M. lysodeikticus*), there appears a zone of complete inhibition of growth around the tissue or secretion. It has been shown also that after a week or two there may be developed in the inhibition zone a few colonies of the microbe, and if these colonies are subcultured they are found to be more resistant to the lytic action of the tissue or secretion than was the original culture. Not only are they more resistant to the lytic action of the tissue or secretion with which they have been grown in contact, but they are equally resistant to all other tissues or secretions; for instance, colonies which have developed in the neighbourhood of a piece of tonsil or cartilage have acquired as much resistance to tears or egg-white as they have to the tonsil or cartilage. Thus in one operation there has been developed a strain of the microbe relatively resistant to the bacteriolytic action of tissues.

Is this Acquired Resistance to Lysis Temporary or Permanent?

When originally isolated, strains of *M. lysodeikticus*, which had by the method outlined above been made resistant to various tissues and secretions, were tested with egg-white, and their susceptibility to lysis by this substance was noted. Nine months afterwards the same strains, which in the meantime had been repeatedly subcultured at intervals of 1 or 2 weeks, were again tested with egg-white in the same way. The results obtained are set forth in Table I.

It will be seen from this table that at the end of 9 months the "resistant" strains had not changed in character, but were just as resistant to lysozyme as they were when first isolated.

Development of Highly Resistant Strains of Bacteria.

Attempts to develop a highly resistant strain were made with *M. lysodeikticus* and also with a *Streptococcus fæcalis*. The method used was to plant the microbes thickly into broth containing that dilution of egg-white which just completely dissolved the microbes in 24 hours at 45° C. (as judged by naked-eye observation). In the case of *M. lysodeikticus* this was a 1 in 350,000 dilution and with streptococcus 1 in 4000 dilution of the egg-white. Although

in these concentrations almost all the bacteria were dissolved, some of them survived and grew out, and these were again subcultured into stronger and stronger concentrations of egg-white in broth. In this way the cultures were made more and more resistant to the bacteriolytic action of egg-white, so that in the case of *M. lysodeikticus*, the culture which originally was completely dissolved in 3 hours by egg-white 1 in 64,000 was after 91 weeks only dissolved by egg-white 1 in 800, while the streptococcus which originally was completely dissolved by a 1 in 800 dilution of egg-white was after 67 weeks' treatment only partially dissolved by a 1 in 5 dilution.

TABLE I.—*Illustrating the Permanence of the Resistance to Bacteriolysis of* M. lysodeikticus *Grown in the Neighbourhood of Tissues and Secretions.*

Tissue to which strain was made resistant.	Lowest concentration of egg-white which in 3 hours at 45° C. showed *any* lysis of a suspension of the bacteria.	
	When first isolated.	After 9 months.
Tears	1 in 243,000	1 in 243,000
Nasal mucus	1 „ 243,000	1 „ 243,000
Liver	1 „ 243,000	1 „ 243,000
Kidney	1 „ 243,000	1 „ 243,000
Spleen	1 „ 81,000	1 „ 243,000
Stomach	1 „ 243,000	1 „ 243,000
Tonsil	1 „ 243,000	1 „ 243,000
Cartilage	1 „ 243,000	1 „ 243,000
Control (non-resistant)	1 „ 2,137,000	1 „ 2,137,000

Table II shows the gradual increase in the resistance to lysis by egg-white of cultures treated in the above manner:

TABLE II.—*Showing the Gradual Increase of Resistance to Lysis of Bacteria Grown in Increasing Concentrations of Egg-White.*

Organism.	Number of weeks of growth in egg-white broth.	Lysis shown in 3 hours at 45° C. by egg-white in dilutions as under—											
		1/800.	1/1000.	1/1600.	1/2000.	1/3200.	1/4000.	1/6400.	1/8000.	1/12,800.	1/16,000.	1/32,000.	1/64,000.
M. lysodeikticus	Before	+	+	+	+	+	+	+	+	+	+	+	+
	2	...	+	...	+	...	+	...	+	...	+	+	AC
	10	...	+	.	+	...	+	...	+	...	AC	−	−
	65	+	...	+	...	+	...	AC	...	±	...	...	...
	91	+	..	AC	...	±	...	−	...	−	...	...	...
		1/5.	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.	1/800.	1/1600.	1/3200.	1/6400.
Streptococcus	Before	+	+	+	+	+	+	+	+	+	AC	AC	±
	8	+	+	+	+	+	+	AC	±	±	−	−	−
	28	...	AC	AC	AC	AC	AC	AC	±	−	−	−	−
	67	±	?	?	−	−	−	−	−	−	−	−	−

+ = solution; AC = almost complete; ± = slight; − = no solution.

Bactericidal Power of Egg-White to "Resistant" and "Non-Resistant" Strains of Streptococcus.

It has been shown above that by growing streptococci in egg-white they acquire the power of resisting the bacteriolytic action of this substance. Experiments were done to see how far they had acquired a resistance to the bactericidal power of egg-white.

A series of dilutions of egg-white were made, and to 10 c.mm. of each of these dilutions was added 10 c.mm. of a suitably diluted broth culture of the resistant and the non-resistant streptococci. These were incubated for 1 hour at 37° C. and then plated on agar.

The results are shown on Table III:

TABLE III.—*Bactericidal Power of Egg-White to "Resistant" and "Non-Resistant" Strains of Streptococcus.*

Dilution of egg-white.	Number of colonies of streptococci.	
	Non-resistant strain.	Resistant strain.
1 in 2	9	153
1 in 4	29	166
1 in 8	56	203
1 in 16	117	174+
1 in 32	136	...
Control (no egg-white)	135	202

It will be seen from these figures that the resistant strain has acquired a definite well-marked increase of resistance to the bactericidal power of egg-white.

Does a Streptococcus which has been made Resistant to Egg-White offer any Increased Resistance to the Bactericidal Power of Human Blood?

To settle this question bactericidal experiments were done with resistant and non-resistant strains in slide-cells. Serial dilutions of a broth-culture of both strains were made, and 2·5 c.mm. of each of these dilutions were added to 50 c.mm. of human defibrinated blood and placed in slide-cells. The results obtained were as shown in Table IV:

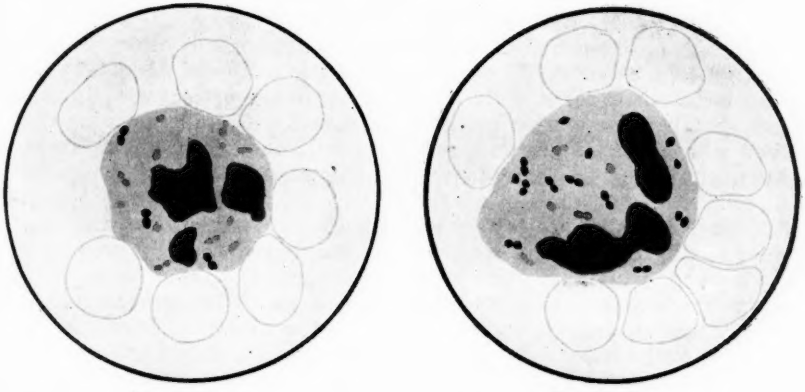
TABLE IV.—*Bactericidal Power of Human Blood on Streptococci which had been made Resistant to Egg-White.*

	Number of colonies developing in blood planted with dilutions of broth-culture as under.			
	1/1.	1/10.	1/100.	1/1000.
Streptococcus J:				
Resistant	+	206	52	10
Non-resistant	+	32	0	0
Streptococcus A:				
Resistant	+	112	19	0
Non-resistant	+	5	0	0

The figures given in Table IV show that there is a very definite diminution of the susceptibility to the bactericidal power of human blood of two strains of streptococci, which had been made resistant to the bacteriolytic power of egg-white.

Is an Egg-White Resistant Strain of Streptococcus less susceptible to Intracellular Digestion by Leucocytes?

It has been shown by many experiments that the bactericidal power of human blood to streptococci is largely a function of leucocytes, and in all probability is mainly dependent on phagocytosis and intracellular digestion of the microbes. Having shown that the strains of streptococci which had been made resistant to egg-white are less susceptible to the bactericidal power of



Non-resistant. Egg-resistant.

Typical leucocytes after incubation of streptococci with human blood for 3 hours at 37° C.

FIG. 1.—Intracellular digestion of non-resistant and egg-resistant fæcal streptococci.

the blood, it was interesting to see whether they were also less susceptible to intracellular digestion than were the normal cocci. Bacterial suspensions of agar cultures of the normal and resistant strains were made. These were mixed with equal volumes of normal human defibrinated blood and incubated for 3 hours at 37° C. in capillary tubes, after which films were made and stained, and the state of the bacteria inside the leucocytes was observed. There was found to be much more intracellular digestion of the normal strain than there was of the resistant strain. It is, of course, impossible to express this numerically, but whereas with the non-resistant strain there were only a few normally staining cocci inside the leucocytes, with the resistant strain there were many well-stained cocci and comparatively few shadows. Fig. 1 shows typical examples of leucocytes filled with each type of coccus, and the difference in the appearance of the resistant and the non-resistant strains is obvious.

This phagocytic experiment was made in 1924, and the cultures were put aside and not opened for two years, when subcultures were made and a similar phagocytic experiment was performed with the same result, showing that the streptococci which had been made resistant to the lysozyme of egg-white, had maintained their resistance to intra-cellular digestion by human leucocytes for two years.

From these results it would appear that there may be some connection between the bacteriolytic power of egg-white and the power of leucocytes to digest microbes which have been phagocyted; in other words, the intracellular digestion of bacteria by leucocytes may be due to a ferment similar to that which we have described as occurring in many animal secretions and tissues, and which we have called lysozyme.

DISCUSSION.

The observations detailed above show that by growing bacteria in egg-white, cultures can be obtained which are resistant not only to the bacteriolytic and bactericidal action of the egg-white, but also to the bactericidal power of human blood and to intracellular digestion by human leucocytes. It has been shown that bacteria can be made resistant to egg-white by growing them in contact with human tissues and secretions, and although the direct experiment has not been made, it is extremely likely that these bacteria grown in contact with human tissues are also resistant to the bactericidal power of the blood and to leucocytes. It is also very probable that the virulence of the microbes could be enhanced in this way, and it was hoped that by growing these bacteria in egg-white they would be rendered virulent, but even at the end of the experiments they were still non-virulent for animals. It must be remembered, however, that the two microbes chosen for the experiment were both very susceptible to the lytic action of the tissues, and were quite avirulent, and it is possible that had microbes been chosen which were slightly pathogenic for animals, similar treatment would have resulted in an increase in their virulence. Murray (1924) has shown that by growing meningococci in contact with leucocytes the virulence of the cocci is increased, and it is probable that this is due to an enhanced resistance to lysozyme as in the experiments detailed above.

SUMMARY.

Bacteria grown in contact with tissues or secretions are rendered comparatively resistant to their antibacterial action.

This resistance to lysozyme action is not specific, in that bacteria made resistant to one tissue are equally resistant to all tissues and secretions.

By repeated subculture into stronger and stronger dilutions of egg-white bacteria were made to acquire considerable resistance to the bacteriolytic action of this substance. At the same time, however, they acquired resistance to the bactericidal power of the blood and to intracellular digestion by leucocytes.

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TETANUS.—III. NON-TOXIC VARIANTS OF *B. TETANI*.

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In a previous paper (Fildes, 1925[2]) it was shown that no less than 30 out of 75 strains of agglutinating tetanus bacilli, recently isolated, were non-toxic even in doses of 1 c.c. into mice. This result was remarkable in that neither Tulloch nor Tenbroeck and Bauer had recorded non-toxic strains among the large number they had isolated.

Further confirmatory work has been carried out with the following results:

RECOVERY OF NON-TOXIC STRAINS FROM CASES OF TETANUS.

Eleven further cases of tetanus in man have been examined, and from these 10 toxic strains were recovered and one non-toxic. One of the toxic strains had only a feeble pathogenic action. Of the 17 cases, therefore, which have been examined to date, 16 showed evidence of toxicity to a varying degree and one was non-toxic.

The actual results with 6 consecutive cases were as follows:

Toxicity Tests with Filtered Cultures.

The 6 strains were grown for 10 days in broth containing digested blood (influenza medium, Fildes, 1920). They were then filtered and injected into guinea-pigs (thigh) in doses falling from 1 c.c.

Strain.	M.L.D.	Time of death.
TM 8	1/10,000 c.c.	3 days.
TM 9	*Nil*	...
TM 10	1/10,000 c.c.	3 days.
TM 11	,,	5 ,,
TM 12	*Nil*	...
TM 13	1/10,000 c.c.	5 days.

Toxicity Tests with Spores.

The 6 strains were grown for 5 days on "influenza" plates, and then made with saline into suspensions of equal opacity and heated to 65° C. for 1½ hours. Four dilutions were then made with saline, and 0·5 c.c. of each injected as above with 0·5 c.c. of a 10% suspension of earth, autoclaved.

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Strain.	M.L.D.; dilution of suspension.	Time of death.	Profusion of growth.	Morphology of spores.
TM 8	1/10,000	7 days	+++	Many "ideal."
TM 9	*Nil* (in 1/1)	...	+	Very few; "sphenoides" types; some large and bulbous with long filaments.
TM 10	1/10,000	4 days	+++	Many "ideal."
TM 11	1/100	4 „	++	Numerous "ideal"; somewhat filamentous.
TM 12	1/1	5 „	++	Few "ideal"; filamentous.
TM 13	1/1000	4 „	+++	Many "ideal."

These results both show that TM 8 and TM 10 were the most toxic, followed by TM 13 and TM 11. TM 12 showed evidence of toxin-formation with spores only, while TM 9 was ineffective by both methods.

Furthermore, there is seen to be a definite parallelism between toxicity and profusion of growth, and equally a parallelism between toxicity and "ideal" morphology. As the toxicity falls the spores decrease in number and the bacillus tends to become filamentous. Objection may be taken to this conclusion on the ground that in the non-toxic strains the number of spores is small, and thus the actual doses of viable spores injected will be smaller than in the toxic. This fact undoubtedly prevents any precise quantitative comparison, but is far from sufficient to account for the demonstrated differences in toxicity. A dilution 1/10,000 of TM 8 contains much fewer spores than a 1/1 suspension of TM 9. Further, a dilution 1/10,000 of TM 9 still contains viable spores which actually vegetate in the lesion. This strain, therefore, which vegetates both *in vitro* and *in vivo* and yet produces no demonstrable toxin in either situation, exhibits no specimens with ideal morphology; in fact the appearance has become so "abnormal" that it cannot be recognized as a tetanus bacillus at all. Furthermore, the surface-culture shows little tendency to develop the long filamentous edge described as characteristic of tetanus bacilli (Fildes, 1925[2]), and thus the organism can only be distinguished from strains known as *B. cochlearius* or *B. putrificus* by the fact of agglutination with tetanus serum.

It is possible that this tendency of non-toxic strains to variation from the toxic ideal morphology—a tendency already noted (Fildes, 1925[1])—may to some extent account for the absence of such strains from the collections of other workers. Bauer and Meyer (1926), for instance, when isolating tetanus bacilli from human stools, prepared the customary mixed culture and examined it microscopically after 4–6 days. "After some experience it is relatively easy to recognize tetanus bacilli among other round or oval terminal spore-bearing organisms which are commonly present in these cultures. Those which were found positive for tetanus-like organisms were transferred"—for isolation and found toxic. In the case of TM 9 there was nothing in the mixed culture to suggest to me that tetanus bacilli were present, and it was only the routine culture on solid medium which disclosed a film-growing strain which agglutinated with type I serum.

ISOLATION OF NON-TOXIC STRAINS FROM TOXIC.

On several occasions it has been noted that cultures taken from toxic stocks preserved in the laboratory have been non-toxic. These stock cultures had been unsealed and subcultured by the film method from time to time as they dried up, to check their continued purity.

It was thus obvious that under certain circumstances a non-toxic strain might be separated from a specimen which contained an apparently pure toxic strain.

This question was studied as follows:

Material was taken direct from a case of human tetanus, and two samples of earth which contained tetanus bacilli were selected. These were inoculated into Robertson's meat medium, heated to 65°C. for 1½ hours and incubated for 4 days. One drop of each of these cultures was placed at the bottom of a dry tube of "influenza medium," and the next day the resulting film was subcultured from as low down the surface as possible without disturbing the contaminating organisms. The cultures so obtained were pure tetanus bacilli, the human strain being type I and the two earth strains each type V. The whole slopes were then transferred to Robertson's medium for preservation. These latter cultures after three days' growth killed guinea-pigs overnight in doses of 0·5 c.c.

The subsequent experiments were then conducted as follows:

One drop of the Robertson culture was placed at the bottom of a sloped tube of "influenza" medium. When this (culture A) had grown, a subculture was made from one of the ultimate filaments to the bottom of another tube, and subsequently every one to three days again from filament to bottom on each occasion (top series). Another subculture was made from the bottom of culture A to the bottom of another tube, and so on from bottom to bottom (bottom series).

The M.L.D. for guinea-pigs was determined for culture A, and on two other occasions for the top and bottom series. One drop of a suspension of the whole slope to be tested was transferred to broth, and after 10 days these cultures were injected intramuscularly (thigh).

Strain TM 17 (man).

M.L.D. of culture A						0·001	in 6 days.
„ „ bottom series	after	3	subcultures			0·001	„ 4 „
„ „ „ „	„	6	„			0·001	„ 3 „
„ „ top	„	3	„			0·001	„ 4 „
„ „ „ „	„	6	„			0·001	„ 4 „

Strain TS 39.

M.L.D. of culture A						0·01	in 2 days.
„ „ bottom series	after	3	subcultures			0·01	„ 2 „
„ „ „ „	„	6	„			0·01	„ 2 „
„ „ top	„	3	„			0·001	„ 5 „
„ „ „ „	„	6	„			0·01	„ 2 „

Strain TS 40.

M.L.D. of culture A									0·001	in 2 days.
,,	,,	bottom	series	after	3	subcultures			0·001	,, 2 ,,
,,	,,	,,	,,	,,	6	,,			0·001	,, 3 ,,
,,	,,	top	,,	,,	3	,,			0·01	,, 8 ,,
,,	,,	,,	,,	,,	6	,,			0 (1 c.c.=*nil*).	

Thus, while in two cases subcultures from filaments had no effect on toxicity, in the third case the strain became non-toxic, although the parallel series of cultures from the bottoms of the slopes remained unaffected.

No constant difference was observed between the two strains in their morphology, cultural or serological reactions.

RELATION OF THE NON-TOXIC TO THE TOXIC STRAIN.

It is clear that apparently pure cultures isolated by the film method may really contain strains with different characters. This, of course, is not surprising, since all varieties present in the original sample would grow intermingled in the film, but in practice it is very unusual to find two strains together which are so different as to give different serological reactions. Excluding the cases of cross-agglutination between types II and III, and types IV and V, I have only six times observed double agglutinations, four times between types II and V, and twice between types I and III. On the other hand, if different serological types are artificially mixed together, there is no difficulty in obtaining agglutination of each with the appropriate sera. This rarity of obviously mixed cultures is no doubt due to a relatively feeble concentration of spores in soil or elsewhere.

The case of the non-toxic varieties present in a toxic culture appears, however, to be different. On the seven occasions on which I have separated non-toxic strains from a toxic culture, the non-toxic has always been of the same serological type as the toxic, and furthermore, during the process of separating the non-toxic variety, strains with intermediate toxicity have been encountered. It thus appears more probable that the non-toxic strains are variants of the toxic strains in the culture, rather than a chance contamination by a different variety.

In an attempt to prove that non-toxic variants could be produced by toxic strains, four single-cell cultures isolated by Tenbroeck were subjected to the same procedure as already described, but without success. On the other hand I have separated a non-toxic variety from No. 279 of the National Collection of Type Cultures. This is a very old culture of American origin, which was purified by Miss Robertson in 1917 by twice subculturing two rods by Barber's method. A second sample of this strain from a culture dated 1919, however, did not react in this way.

The failure to detect non-toxic strains in the four single-cell cultures does not appear fatal to the suggestion that they are variants, since there is an element of chance involved, as shown by the failure with one sample of No. 279. It is also quite possible that a single-cell culture might be more stable than a culture freshly isolated from earth in which it had been subjected to the modifying action of light and air.

It may be remarked that no return of toxicity has been observed in any non-toxic strain.

The question now arises as to why the film method shows a propensity for producing non-toxic variants.

There is no evidence that the film growth of a toxic strain is essentially different in speed of spread or in appearance from that of a non-toxic. There seems, therefore, no reason to suppose that the fact of growing in a film renders a strain non-toxic, nor that the non-toxic is more readily separated because it spreads more rapidly as a film. On the other hand, careful examination of the filamentous edge where it encroaches upon the hard dry apex of the slope (Fildes, 1925[1], Figs. 1–8) shows that each filament travels for a variable but very considerable distance without contact with its neighbours, and thus each filament represents the continuous spread of a pure strain. A subculture from one filament is thus equivalent to a single colony obtained by plating out a suspension of single organisms. It is thus a means of rapidly producing generations from single cells and so providing optimum conditions for the selection of variants.

The situation on the other hand is entirely different with isolations of anaërobes by shake cultures. With these it is notoriously difficult to obtain colonies from single cells; indeed, it is sometimes necessary to make repeated shake subcultures over a period of weeks before the tetanus bacilli can even be separated from contaminants. The difficulty is so great that a number of authors have recorded their inability to isolate *B. tetani* at all.

Under these circumstances it may be suggested that the absence of non-toxic strains from the series of other workers may be due in part to the inability of a shake culture to provide cultures from single cells unless, perhaps, after attempts more numerous than these authors had reason to make.

An explanation along these lines is strongly supported by Heller's (1922) results. Taking the standard toxin-producing strain of Parke Davis she was able by repeated subculture in shakes to separate no less than six non-toxic varieties. Thus it seems that the method of isolation by shakes does not differ from that of isolation by films, except in that by the former it is much more difficult to divide a culture into its ultimate constituents.

From the practical point of view it was thought that the large number of non-toxic strains previously recorded might readily be due to the accidental selection of non-toxic variants of a toxic strain owing to the large number of subcultures made, and the care with which these were taken from ultimate filaments in order to ensure purity. It was thought that if, during isolation, the subcultures were made from as low down the slope as possible, and if these were reduced to a minimum, samples of earth, for instance, might be shown to contain toxic bacilli, whereas previously only non-toxic had been found.

The number of subcultures was reduced by using for enrichment a medium modified from Tulloch's (1917–19), using fresh meat in the manner of Robertson's medium instead of kidney, and omitting the formate. Such a medium has a similar specific effect for tetanus and related bacilli as Tulloch's.

Twenty-seven samples of earth were inoculated into this medium, heated for 1½ hours at 65° C., and incubated for 4 days. One drop was then transferred to the bottom of a sloped tube of "influenza medium," and after

2 days' incubation the films which grew were subcultured from low down the slope and the eventual pure culture tested for toxicity. Seventeen strains were thus obtained, of which only 3 were non-toxic, whereas formerly 20 were non-toxic out of 33. On the other hand, 55 samples of cow-dung were examined and 44 found positive, of which 23 were non-toxic, which is a greater proportion than formerly found among horses (9 out of 34 non-toxic).

The total result to date with earth shows 23 non-toxic out of 50 strains, or about 50 %, and in cows the figure is similar. There is thus little evidence that the possibility of selecting non-toxic strains by the film method has any important bearing upon the frequency with which non-toxic strains are isolated in practice.

It is concluded that specimens of dung or earth very frequently contain tetanus spores which, though capable of vegetating *in vivo*, do not produce toxin. Further, such specimens may contain toxic spores of such a character that less toxic variants and totally non-toxic variants are readily split off. Stock cultures may also under certain conditions undergo changes in a similar direction.

With regard to material from cases of tetanus, it is inconceivable that non-toxic strains can be present alone. In the one example noted the specimen examined was merely a cotton-wool swab sent through the post, whereas all others were fragments of tissue or indeed the whole lesions. It is therefore quite possible that the toxic strains present in the wound did not come in contact with the swab, or if they did, became modified during passage through the post. In any case it is clear that non-toxic variants may exist even in cases of tetanus.

DISSOCIATION AMONG TETANUS BACILLI.

The demonstration of a spontaneous change from toxicity to lack of toxicity and the splitting off of non-toxic variants is not the only evidence of that fluidity of character which has recently been reviewed by Hadley (1927) under the heading " microbic dissociation." Since, however, Hadley made little reference to this organism, the following data may be enumerated :

Morphology.—It has been indicated that strains may be said to have an ideal morphology when they approximate to the classical description, but that others may be progressively more abnormal until they would not ordinarily be recognized as being tetanus bacilli at all. The former tend to be toxic and the latter non-toxic, although strains with an ideal morphology may also be non-toxic.

Cultures.—The great majority of recently-isolated strains grow as a rapidly-spreading film, whereas some old cultures and the non-motile strains tend to produce isolated colonies. These differences are akin to the " H " and " O " *proteus* strains, but are not related to toxicity. The quality of the edge of the spreading culture, however, does show some relation to toxicity, the long-filament variety being more often toxic than the short.

Motility.—Whereas practically all strains are actively motile, a few non-motile varieties have been isolated by Bauer (1924). Presumably this character also is not immutable, since I have once obtained a motile subculture from one of Bauer's non-motile strains.

Agglutination.—The agglutination of all " H " forms of tetanus bacilli is

very rapid and of the large flocculant variety. On the other hand, that of the "O" (non-motile) forms is feeble, requiring at least 5 hours at 56°C., and is fine and granular.

Agglutination with salt solution.—Strains of type III, whether toxic or not, have a marked tendency to spontaneous agglutination in salt solution, and even in broth cultures sediment more readily than other varieties.

Biochemical reactions.—Non-toxic strains often do not exhibit the rapid gelatine-liquefying function of the toxic strains. This character is the more marked the more widely they diverge from the ideal.

All these points are illustrated in the S–R variation observed among other bacteria, but in the case of *B. tetani* it is seldom that the occurrence of this variation can be detected in any particular strain except in the loss of toxicity. The strain No. 279 in the National Collection does, however, develop "O" characters on keeping, which revert to "H" on suitable subculture.

The chief interest in these evidences of dissociation is the bearing they have on the relation between *B. tetani* and allied organisms. *B. tetanomorphus* (or *tetanoides ?*) is stated to differ from *B. tetani* in its saccharolytic activity and absence of protein-splitting property. On the other hand *B. putrificus* (or *cochlearius ?*) is stated to have greater proteoclastic action than *B. tetani*. Both of these organisms come within the range of variation in morphology of *B. tetani*, and also exhibit the same tendency to develop surface films to a greater or less extent. *B. tetanomorphus* is stated by McIntosh (1917) to ferment glucose and maltose, but I have come across strains of similar bacilli which have fermented also lactose and saccharose. Further, *B. tetani* itself is not totally devoid of action on sugars. Occasional strains are met with, which sometimes produce a small quantity of acid in glucose (Fildes, 1925[1]), and according to Reddish and Rettger (1924) there is always a considerable consumption of glucose, although not enough to change the colour of an indicator. The same workers also credit *B. tetanoides* with considerable peptolytic action.

B. putrificus, on the other hand, according to Reddish and Rettger, has a marked, though delayed, digestive action on coagulated protein. This is not, however, observed with *B. cochlearius*, or with a number of similar organisms which are encountered in soil and fæces. At most these organisms slightly disintegrate meat, and soften coagulated serum to the same extent as *B. tetani*.

There appears, therefore, to be a large series of organisms whose characters in common are a tendency to produce a terminal spherical spore and a surface film growth. At one extreme of this series there is a marked saccharolytic activity and feeble proteolytic, at the other a feeble or absent saccharolytic and a more pronounced proteolytic. *B. tetani* occupies a central position, and tends to merge on each side into the behaviour of its neighbours. There is no absolute distinction between this bacillus and its relatives, except the possibly artificial one of agglutination with sera made from those strains which produce the characteristic toxin.

In routine work with this series it becomes clear that those organisms, other than *B. tetani*, which have been invested with specific names are, perhaps, unworthy of them, being at best merely representatives of the saccharolytic extreme on the one hand, or the proteolytic on the other.

CONCLUSIONS.

1. Confirmatory evidence is produced of the existence of non-toxic strains of *B. tetani* in soil or dung, and even in a case of tetanus.

2. These strains are looked upon as variants from the "ideal" tetanus bacillus.

3. Evidence of variation in other characters of the tetanus bacillus is noted.

4. Variation in all characters is so marked that the more extreme examples cannot be differentiated from related organisms except by agglutination.

5. *B. tetani* and certain named and unnamed species constitute a biological group between the various members of which there is no absolute distinction.

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TETANUS.—IV. THE GEOGRAPHICAL INCIDENCE OF TETANUS IN ENGLAND AND WALES.

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TETANUS is responsible for a relatively minute proportion of the general death-rate of England and Wales, as, although the disease is usually fatal, it occurs only occasionally. In view of present knowledge regarding the usual sources of infection, it is probably more or less generally known that tetanus is more common in the male than in the female sex, and is predominantly a rural disease, but comparatively little is known regarding geographical variations in the mortality due to it. In connection with Dr. Fildes's series of studies of tetanus, a knowledge of its varying prevalence throughout the country was essential, and the present paper contains the results of an analysis of such data as were available in an attempt to elucidate this subject.

MATERIAL FOR INVESTIGATION.

The registered deaths from the disease were obtained from the annual reports of the Registrar-General, and from other original records which were transferred, for preservation, to the Statistical Department of the Medical

Research Council a few years ago by the Registrar-General. The mortality from tetanus in its present-day concept cannot be traced further back than the beginning of this century. Up to the year 1900 deaths from the disease were tabulated under the heading "idiopathic tetanus," which excluded cases of tetanus due to injury. In 1900 trismus neonatorum was first classed with tetanus, and in 1902 tetanus as a cause of death was, for the first time, given preference over other causes mentioned on the same certificate for purposes of tabulation, with the result that thereafter the annual number of deaths ascribed to the disease was practically quadrupled. The registered deaths from tetanus as a whole in the individual registration counties of England and Wales have only been published for the separate sexes at all ages in the annual reports of the Registrar-General for each of the ten years 1901–10, inclusive, as it is not included in the selected list of causes of death of which particulars for the respective counties are tabulated in the annual reports for subsequent years. With the object of investigating the topographical variation in the mortality from the disease, the mean annual crude mortality rates for the respective counties for this decennial period were first calculated from these data. The original records already referred to give in detail, however, the deaths from the disease by ages in the registration counties and their sub-divisions, registration districts, for the nine years' period 1901–09, the records for 1910, the last year of the decade, being unfortunately not available. As it was evident from reference to these records that the distribution of deaths at the several ages in some of the registration counties showed definite variation from that obtaining in the country as a whole, and as the age distribution of the population in the counties that are predominantly rural is known to differ from that in counties that may be described as urban in character, it was deemed advisable to calculate standardized death-rates from the disease in the separate sexes for the counties. These standardized death-rates and the numbers of deaths on which they are based are shown in Table I. To facilitate the study of the varying geographical incidence of the disease, two maps were drawn, one for males and another for females, showing by a scheme of cross-hatching the topographical distribution of the rates that fell into the several divisions of an arbitrary scale of magnitude indicating certain degrees or grades of mortality. From the maps it was at once evident that the distribution of excessive mortality in the two sexes was very similar, suggesting that the predisposing causes were local rather than occupational. In some counties the proportion of the mortality due to infantile tetanus, *i. e.* at ages under one year, was very considerable, as will be referred to more fully later, and as the prevalence of tetanus in infancy was unlikely to be influenced to a like degree by the factors that predispose to tetanus in later life, a decision was made to re-calculate the standardized death-rates, excluding the deaths under one year. These death-rates for the separate sexes and for persons as well as the death-rates under one year in the respective counties are also given in Table I, and the map which is reproduced shows the distribution by county or county-group unit areas of the different grades of mortality from tetanus in persons at ages over one year. Mortality-rates are not given in Table I for every county. In two instances relatively small counties with few deaths have been associated with adjacent larger counties. Thus, Rutland, in which one death only from

Table I.

Nos. in map.	Registration counties or groups of counties.	Males. Mean annual death-rate per million. All ages.	Ages 0–1 year.	Ages 1 year and upwards.	Males. Total number of deaths in 9 years—1901–09. Ages 0–1 year.	Ages 1 year and upwards.	Females. Mean annual death-rate per million. All ages.	Ages 0–1 year.	Ages 1 year and upwards.	Females. Total number of deaths in 9 years—1901–09. Ages 0–1 year.	Ages 1 year and upwards.	Persons. Mean annual death-rate per million.	Nos. in map.
	England and Wales	9·7*	105	7·3*	374	1069	2·9	57	1·7	202	265	4·5	
	Urban Group of Counties	7·8	89	5·7	179	442	2·5	49	1·3	97	115	3·5	
	Rural Group of Counties	12·4	96	10·3	40	196	4·1	56	2·9	23	60	6·6	
	Rural Group of Counties (Eng. Section)	13·3	90	11·4	30	173	4·8	70	3·3	23	54	7·4	
1	London	11·7	185	7·1	87	146	3·7	95	1·6	44	34	4·4	1
2	Surrey	12·4	162	8·9	12	30	3·3	55	2·0	4	8	5·5	2
3	Kent	17·1	227	12·1	21	51	6·0	144	3·0	13	13	7·6	3
4	Sussex	10·2	56	9·2	3	23	1·7	58	0·6	3	2	4·9	4
5	Hampshire	10·8	88	9·0	7	32	4·8	89	2·9	7	11	6·0	5
6	Berkshire	19·0	147	15·7	4	20	7·6	150	4·7	4	14	10·2	6
7	Middlesex	13·4	216	8·3	22	33	4·8	139	1·8	14	8	5·1	7
8	Hertfordshire	18·8	125	16·3	3	18	4·2	0	3·6	0	5	10·0	8
9	Buckinghamshire	29·0	394	20·2	7	16	4·8	177	1·1	3	1	10·7	9
10	Oxfordshire	15·9	233	10·9	4	9	11·4	116	8·9	2	8	9·9	10
11	Northamptonshire	10·2	57	9·0	2	14	3·7	59	2·4	2	4	5·7	11
12	Huntingdonshire	19·1	223	14·0	1	3	9·2	231	4·9	1	1	9·5	12
13	Bedfordshire	15·4	115	12·8	2	10	2·4	59	1·2	1	1	7·0	13
14	Cambridgeshire	29·8	101	26·8	2	25	7·4	103	5·0	2	5	15·9	14
15	Essex	15·0	185	10·9	24	55	3·2	93	1·1	12	6	6·0	15
16	Suffolk	10·4	83	8·8	3	14	5·3	0	5·2	0	9	7·0	16
17	Norfolk	14·0	64	12·3	3	26	5·4	87	3·6	4	8	8·0	17
18	Wiltshire	8·3	78	7·0	2	8	2·4	0	2·7	0	3	4·9	18
19	Dorsetshire	17·7	219	13·2	4	12	2·2	0	1·3	0	2	7·3	19

17 . Norfolk	14·0	64	12·3	3	26	3·4	87	3·6	4	8	8·0	17
18 . Wiltshire	8·3	78	7·0	2	8	2·4	0	2·7	0	3	4·9	18
19 . Dorsetshire	17·7	219	13·2	4	12	2·2	0	1·3	0	2	7·3	19
20 . Devonshire	13·7	169	9·9	10	29	4·6	85	2·7	5	9	6·3	20
21 . Cornwall	15·1	0	15·2	0	20	2·7	0	2·9	0	4	9·1	21
22 . Somersetshire	10·1	95	8·3	4	16	6·4	168	3·2	7	7	5·8	22
23 . Gloucestershire	15·7	95	13·4	6	36	4·2	82	2·4	5	8	7·9	23
24 . Herefordshire	8·2	101	5·5	1	3	0	0	0	0	0	2·8	24
25 . Shropshire	7·7	39	7·0	1	8	0·8	39	0	1	0	3·5	25
26 . Staffordshire	5·1	45	4·1	7	23	1·9	46	0·9	7	5	2·5	26
27 . Worcestershire	10·2	132	7·3	7	16	0·4	0	0·4	0	1	3·9	27
28 . Warwickshire	7·1	28	6·7	3	27	1·6	19	1·1	2	5	3·9	28
29 . Leicestershire with Rutlandshire	9·6	62	8·3	3	17	2·3	60	0·9	3	2	4·6	29
30 . Lincolnshire	9·4	56	8·3	3	19	2·9	38	2·2	2	5	5·3	30
31 . Nottinghamshire	6·2	41	5·3	3	15	3·2	68	1·8	5	5	3·6	31
32 . Derbyshire	6·5	34	5·9	2	13	1·7	54	0·4	3	1	3·2	32
33 . Cheshire	7·6	95	5·6	8	19	1·6	59	0·2	5	1	2·9	33
34 . Lancashire	5·1	45	4·1	22	79	1·6	23	1·1	11	24	2·6	34
35 . Yorkshire (W. R.)	5·0	42	4·1	13	50	1·2	16	0·8	5	11	2·5	35
36 . „ (E. R.)	5·7	77	3·9	4	8	1·8	58	0·4	3	1	2·2	36
37 . „ (N. R.)	7·9	69	6·3	3	11	2·2	47	1·2	2	2	3·8	37
38 . Durham	7·9	175	3·1	29	18	3·1	74	1·3	12	7	2·2	38
39 . Northumberland	12·0	66	10·6	5	30	5·3	54	4·3	4	12	7·5	39
40 . Cumberland and Westmorland	11·8	118	9·2	4	13	3·3	31	2·6	1	4	5·9	40
41 . Monmouthshire	8·2	88	6·1	4	10	2·3	0	2·8	0	4	4·5	41
42 . Glamorganshire	8·6	72	7·0	9	31	1·7	16	1·4	2	6	4·2	42
43 . South Wales	9·2	189	5·0	7	8	1·7	0	1·9	0	3	3·5	43
44 . North Wales	8·3	62	6·9	3	15	1·3	21	0·9	1	2	3·9	44

* As the average annual number of deaths in any county, m, is so small in comparison with, n, its population, the standard deviation of the number of deaths may be assumed to be $\sqrt{m}$, and an approximation to the probable error of the death-rate may be obtained by the use of the formula $\cdot 6745\sqrt{m} \times \frac{1,000,000}{n}$. These errors have not been inserted in the table, but have been considered in referring to significance of differences in the text.

tetanus in each sex was recorded in nine years, was incorporated with Leicestershire, and Westmorland with Cumberland. In the individual rural counties of Wales, excluding the urban county Glamorgan, the numbers of deaths, though available, were so small that death-rates were only computed for two divisional groups—a northern and a southern. From the extremely small numbers of deaths in females on which the county death-rates are based, as shown in Table I, it is obvious that little stress can be laid on the importance of such divergencies as are shown, but the rates were calculated in the expectation that if any general tendency to a regional concentration of mortality were present, it might be revealed.

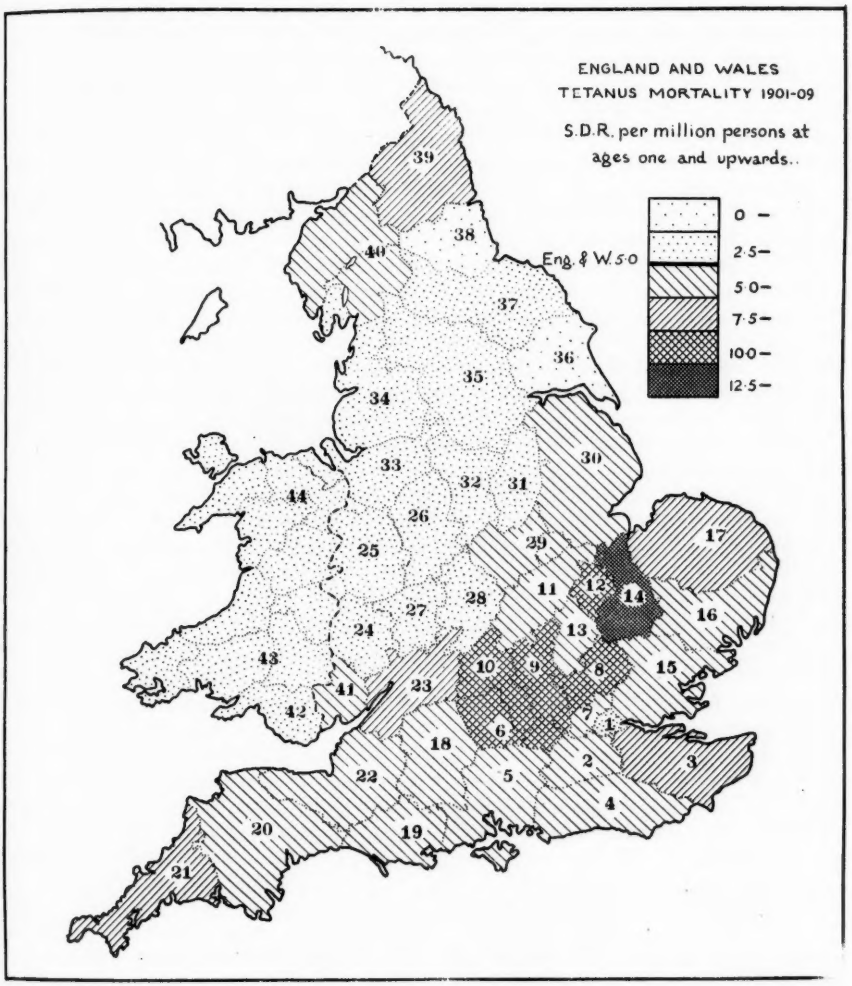
ANALYSIS OF DATA AND DISCUSSION.

From the map on p. 231 illustrating the topographical incidence of mortality from the disease in persons at ages over one year, it will be seen that there is a very definite concentration of the mortality in what may be described as the south midland region of the country, an area comprising Cambridgeshire (14*), Buckinghamshire (8) and the adjacent counties. All the counties lying to the south and east of an oblique line extending from the source of the Humber to the mouth of the Severn, with the exception of London, show a death-rate in excess of that for England and Wales as a whole, whereas the two county divisions comprising the whole extent of Wales and the counties in the remainder of England, with the exception of Northumberland (39), Cumberland with Westmorland (40) in the extreme northern area and Monmouth (41) contiguous to South Wales, show mortality-rates below the level of that for the whole country. Of the counties occupying the region in which the tetanus mortality is especially concentrated, one, Cambridgeshire (14), stands alone in showing the highest grade of mortality depicted in the map—that is, over 12·5 per million persons. Showing the next highest grade of mortality, namely 10 to 12·5 per million, is a group of counties lying adjacent to Cambridgeshire, and including Huntingdonshire (12), Hertfordshire (8), Buckinghamshire (9), Oxfordshire (10) and Berkshire (6). In Bedfordshire, which is almost encircled by this group of counties, and Northamptonshire, which lies adjacent to it, and which, with the others named, complete the south midland group, the rates are lower, but still above the average for the whole country. The next highest grade of mortality, namely 7·5 to 10 per million persons, is found in Norfolk (17) and Gloucestershire (23), lying contiguous to and forming part of the same transverse belt as the group of counties already described as showing the highest incidence and two isolated counties, Kent (3) and Cornwall (21). A similar grade of mortality is shown in the county of Northumberland (39). Among the counties that show mortality rates below the level of the whole country, Durham (38) and the East Riding of York (36) exhibit the lowest values.

The area of highest incidence is so well defined that it seemed as if it might be associated with some particular geological formation. On comparing the distribution with that of a geological map of England and Wales, the counties concerned appeared to be related partly to chalk and partly to oolitic

* The numbers in brackets indicate the numbers of the counties in the map.

formations. Though the soil of the surface varies much more than the solid geological strata because of the presence of soil drifts, the association with a chalk and limestone formation is interesting in view of Bullock and Cramer's



(1919) experimental proof that soluble ionizable calcium salts enable detoxicated *B. tetani* to exert their pathogenic powers, and their conclusion that calcium owes this property to its local action on the tissues. To what extent, if any, a soil overlying a chalk or limestone solid geological formation may be

better provided with substances that enable the tetanus bacillus to exert its pathogenic effect cannot be estimated, but the occurrence of the lowest recorded tetanus mortality-rate in the East Riding of Yorkshire, which rests also for the most part on a chalk formation, indicates that the coincidence of a high tetanus mortality and such a subsoil is not universal, and would suggest that the apparent association may depend on other features of the locality, which will be referred to and discussed more fully later.

Though the numbers of registered deaths from tetanus in the counties were so relatively small, it seemed desirable to pursue the analysis further. In the period under review, 1901–09, the country was divided into registration counties with their subdivisions, registration districts, but in 1911 a new system of tabulation was introduced, in which registration counties were replaced by administrative counties with their subdivisions, urban and rural districts, and the associated county boroughs. As it seemed to be important to try to determine the relative prevalence of tetanus in urban and rural areas of the counties, the deaths in the registration districts comprising the counties were extracted for the period 1901–09, and an attempt was made to classify these into urban and rural groups from their character as defined in the Annual Report for 1911. This was found to be impracticable, as most of the registration districts in which the deaths occurred comprised sub-districts that were both of urban and rural character according to the subsequent classification. Prior to 1911, morever, no correction was made in the deaths registered in, and credited to certain registration districts for deaths of temporary residents such as hospital patients, who might die in the hospital of an urban area to which they had been transferred for treatment from an adjacent rural area where the disease was contracted. Fortunately, however, a distinction can be drawn for the period under review between the counties that were mainly rural and those that were urban in character. The Registrar-General has selected from the counties of England and Wales two strongly contrasted groups, an urban and a rural, the former comprising counties which at the census of 1911 were mainly urban in character, containing the chief centres of industry, and the latter mainly rural in character though containing some urban communities. The mean annual death-rates from tetanus at all ages, at ages 0–1 year, and at ages 1 year and upwards in males and females in these two groups of counties (the members of which are given in the foot-note)* were calculated. They are shown at the upper end of Table I. For males the death-rate at ages over one year in the urban group was

* *Urban Registration Counties.*

Glamorganshire.	Middlesex.	Nottinghamshire.	East Riding (with York),
Lancashire.	Monmouthshire.	Staffordshire.	Yorkshire.
London.	Northumberland.	Warwickshire.	West Riding, Yorkshire.

Estimated mean population 1901–10, 18,403,946.

Rural Registration Counties.

Anglesey.	Carmarthenshire.	Hertfordshire.	Norfolk.	Shropshire.
Brecknockshire.	Cornwall.	Huntingdonshire.	Oxfordshire.	Somersetshire.
Buckinghamshire.	Carnarvonshire.	Lincolnshire.	Pembrokeshire.	Suffolk.
Cambridgeshire.	Denbighshire.	Merionethshire.	Radnorshire.	Westmorland.
Cardiganshire.	Flintshire.	Montgomeryshire.	Rutlandshire.	Wiltshire.

Estimated mean population 1901–10 4,404,686.

5·7 per million, and that in the rural group 10·3 per million, *i. e.* practically double the urban rate. As the selected group of rural counties included eleven Welsh counties, in all of which, taken in two divisions, the death-rates from tetanus were below the average for the whole country, the death-rate in the English group of rural counties was calculated separately. This rate was found to be 11·4 per million, or actually double the urban rate of mortality, so that the mortality from tetanus in males in the rural group of English counties is apparently definitely in excess of that in the urban group. In females at ages over one year, the mean annual mortality-rate from tetanus in the urban group of counties was 1·3 per million, in the complete rural group 2·9 per million, and in the English section of the rural group 3·3 per million. The female mortality in the rural group of counties is thus at least double the mortality-rate in the urban group. Unlike what is found at the higher ages, the mortality-rates from tetanus at ages 0–1 year were practically the same in urban and rural groups of counties as is shown by rural mortality-rates of 96 and 56 per million as against urban rates of 89 and 49 per million in males and females respectively.

Although it has been shown by Dr. Stevenson (1913) for England and Wales that tetanus is more common in rural than in urban surroundings, no attempt has been made to investigate the subject more fully. It seemed not improbable that more definite evidence of an association between the prevalence of tetanus and the rural character of the counties, or the proportion of the population in a rural environment or other allied conditions, might be elicited by the method of correlation. In the first place, the tetanus mortality-rates in males at all ages, and at ages over one year in the 44 counties or county groups, were correlated with the percentage of the total male population of the respective counties at ages over 10 engaged in agricultural pursuits as determined from data in the census of England and Wales for 1911.*

The correlation coefficient for tetanus at all ages was 0·410 $\pm$ 0·125, which indicates a direct and significant association. The corresponding coefficient became 0·445 $\pm$ 0·121 on using the tetanus mortality-rate at ages over one year. The proportion of the male population in the counties engaged in agriculture was then correlated with the county death-rates from tetanus in certain age-groups, namely, 0–1, 5–19, 20–44, 45–64, and 65 years and upwards. A selection of the principal correlation coefficients obtained with their standard errors are shown in Table II. Though the death-rates at these ages are often based on small numbers of deaths, and can, on this account, have little emphasis laid on their stability, it is interesting to note that whereas the tetanus mortality-rate in infancy has no association with the rural character of the counties in so far as this feature can be denoted by the index used, the mortality-rate in all the remaining age-groups is definitely related to this factor or to some allied condition.

Another index of the proportion of the population engaged in agriculture or the rurality of each county, or more precisely defined, the amount of land under cultivation in the counties of England and Wales, is available. This

* Occupational Group, No. VII, Agriculture. On farms, woods and gardens, males occupied and retired, from age 10 upwards. Census of England and Wales, 1911, vol. x, Pt. I, Table 12, p. 125.

TABLE II.—*Correlation Coefficients.*

Variables.	r.
County death-rate from tetanus in males at all ages and percentage of male population at ages 10 years and upwards engaged in agricultural pursuits	+·410 ± ·125
,, ,, ,, at ages 0–1 and percentage of male population at ages 10 years and upwards engaged in agricultural pursuits	+·135 ± ·148
,, ,, ,, at ages 1 year and upwards and percentage of male population at ages 10 years and upwards engaged in agricultural pursuits	+·445 ± ·121
,, ,, ,, at all ages and percentage of cropping and grass-land arable	+·418 ± ·124
,, ,, ,, at ages 0–1 year ,, ,, ,, ,,	+·002 ± ·151
,, ,, ,, at ages 1 year and upwards ,, ,, ,, ,,	+·483 ± ·116
,, ,, ,, at all ages and total number of cattle per 1000 acres	−·291 ± ·138
,, ,, ,, at ages 1 year and upwards ,, ,, ,, ,,	−·321 ± ·135
,, ,, ,, at all ages and total number of horses per 1000 acres	+·023 ± ·151
,, ,, ,, at ages 1 year and upwards ,, ,, ,, ,,	−·021 ± ·151
Percentage of cropping and grass-land arable and total number of cattle per 1000 acres	−·557 ± ·104
,, ,, ,, ,, and percentage of male population engaged in agriculture	+·531 ± ·108
Death-rate from tetanus at ages 1 year and upwards and percentage of land arable when number of cattle per 1000 acres is constant	+·386 ± ·128
,, ,, ,, ,, and number of cattle per 1000 acres when percentage of arable land is constant	−·072 ± ·150
,, ,, ,, ,, and percentage of arable land when proportion of population in agriculture is constant	+·326 ± ·135
,, ,, ,, ,, and proportion of population engaged in agricultural pursuits when percentage of arable land is constant	+·255 ± ·141

criterion is the percentage of the total area of each county, or, excluding mountain and heath, the percentage of the total area of cropping and grass land that is under arable cultivation. These figures are given for the counties for the year 1918 in Howell's (1924) 'Agricultural Atlas of England and Wales.' Though the arable percentages were probably increased in 1918 as compared with 1911, there is no reason to believe that the relative proportions in the counties underwent any radical change, so that the criterion may be considered sufficiently approximate to be applicable to the previous decade. The correlation of the percentage area of grass and cropping land that is arable in the counties with the tetanus mortality-rate for males at all ages gave the coefficient $0{\cdot}418 \pm 0{\cdot}125$, *i. e.* practically identical with that already obtained by the use of the census data. Correlation of the arable percentages with the tetanus death-rate at ages over one year gave a coefficient of the value $0{\cdot}483 \pm 0{\cdot}116$. The substitution of the arable percentages of the total county areas, including mountain and heath, made no appreciable change in the value of the coefficient. Though the tetanus figures are small and may thus be subject to considerable random error, these correlation coefficients seem to confirm the impression that the topographical incidence of tetanus has a direct relation to the extent to which agricultural pursuits are a feature of the different regions.

In the atlas already referred to, the number of horses per 1000 acres and the total number of cattle per 1000 acres are tabulated for the different counties. These data rendered possible the correlation of the regional incidence of tetanus with the local concentration of horses and cattle respectively to which it might bear some relationship. On correlating the county tetanus death-rate in males at all ages, at ages from one year and upwards, and in the different age-groups respectively, with the number of horses per 1000 acres, the coefficients obtained were either zero or quite insignificant. With the number of cattle per 1000 acres the tetanus mortality was negatively correlated. The coefficients between the density of the bovine population and the tetanus mortality in males at all ages and at ages over one year were $-0{\cdot}291 \pm 0{\cdot}138$ and $-0{\cdot}321 \pm 0{\cdot}135$ respectively. These coefficients are significant, and indicate the existence of some inverse association between the variables. There is, however, as might be expected, a high negative association between the number of cattle per 1000 acres and the proportions of arable land in the counties. The apparent association between the concentration of cattle and the prevalence of tetanus seems to be dependent on their common association with the percentage of arable land, because, on applying the method of partial correlation, the coefficient between the percentage of arable land and the tetanus mortality when the proportion of cattle is held constant remains positive and significant, whereas the coefficient between tetanus mortality and the number of cattle is reduced to zero when the percentage of arable land is held constant. The local feature, amongst those examined, most intimately related to the prevalence of tetanus, would thus appear to be the percentage of arable land, or the extent to which the soil is liable to be turned up. That this factor, relationship to agricultural pursuits, to amount of arable land or to rural environment, or by whatever term it may be described, cannot be held to be wholly responsible for the

unequal distribution of tetanus mortality in England and Wales in 1901–09, becomes evident, however, from the fact that there is no correlation between it and the tetanus mortality in males at ages under one year in the counties.

A feature of the mortality from the disease in the period under review was the relatively large proportion of the deaths that occurred in infancy. Of the total registered deaths from tetanus in the whole country, 26% in males and 44% in females occurred at ages under one year, and practically all the deaths under one year, namely, 95% in males and 98% in females, took place in the first three months of life. The proportions of infantile deaths from tetanus in the different counties showed considerable variation in both sexes. In males the percentage varied from 62% (29 out of 47 deaths) in the county of Durham to 7% (2 out of 27 deaths) in Cambridgeshire.

At ages 0–1, the mean annual male mortality-rate from the disease in the country as a whole was 105 per million, and the corresponding rates in different counties varied from zero to 394 per million. The association of tetanus with vaccination sores in this country is practically unknown. Probably most of the infantile tetanus or tetanus neonatorum in the period under review was due to lack of care or cleanliness, or possibly the application of contaminated dusting-powder to the umbilicus. Another possibility, not altogether remote, is that the length of the tetanus death-roll at this age in some counties may be attributed in some measure to errors of diagnosis, deaths from infantile convulsions in some cases being ascribed to tetanus.

For the nine years' period under review the deaths in age-groups were extracted for the 28 registration districts comprising the registration county of London, but as the numbers of deaths in individual districts were small and the distribution of deaths might be determined largely by the location of hospitals, death-rates were only calculated for the districts of London grouped into five regional divisions, namely, east, west, north, south, and central. The death-rates at all ages in these divisions were as follows:

Divisions of London.	Mean annual death-rates per million.		No. of deaths from tetanus in 9 years 1901–09.	
	Males.	Females.	Males.	Females.
North	10·4	1·0	44	5
South	9·8	3·7	76	32
East*	20·4	6·0	67	22
West	8·9	4·1	28	15
Central	11·0	5·2	8	4
London (as a whole)	11·7	3·7	223	78

This suggests a tendency for some concentration of the mortality in the eastern division of London, but the excess over that in the other divisions is probably not sufficiently great to be considered significant.

It seemed to be of interest to ascertain what information could be obtained regarding topographical variations in the distribution of tetanus in England

* East London includes the registration districts of Shoreditch, Bethnal Green, Mile End, Limehouse, Bow, Bromley and Poplar, and West London those of Paddington, Kensington, Hammersmith, Fulham, Chelsea, Westminster and Strand.

and Wales from the data available in the annual reports of the Registrar-General most recently issued. There is evidence that the age-distribution of the deaths from the disease has changed considerably in recent years from that shown in 1901–09. For the seven years' period 1919–25, the percentages of the total deaths from the disease that occurred under one year in the country as a whole were 9 and 14, as against 26 and 44 in males and females respectively in the earlier period. The change that has taken place in recent years in tetanus incidence and age-distribution at death as compared with the period under review is shown in detail in Table III. From this table it will be seen that whereas the mean annual tetanus mortality-rate in males at all ages for the whole country in 1919–25 has fallen to 67% of that in 1901–09, and has declined to a like extent in the age-groups from 20 upwards, the mortality-rate at ages 0–4 in 1919–25 is only 33% of that in the earlier period, though, as already described, a large proportion of the deaths occur at these ages. In the age-group 5–19 years, the mortality-rate shows no decrease in the later period. Comparison of the mean rates of mortality in females in the two periods shows, in the more recent, a relative decline in mortality at all ages, and at ages 0–4 years, almost identical with that shown at the corresponding ages in males. Again there is no decline in mortality at ages 5–19, and the mean mortality at ages 20–44 in females in 1919–25 also remains as high as in 1901–09. Passing to consider in greater detail the data relating to tetanus for the period 1919–25 alone, Table IV shows for this septennium the mean annual rates of mortality from tetanus in the several age-groups in the only sub-divisions of the country for which tetanus figures are published, namely, the aggregate of rural and urban districts respectively comprising the administrative counties, the county boroughs in the aggregate and the administrative county of London, and the death-rates at the several ages in these divisions stated as percentages of the corresponding rates for the whole country. The difference between the mortality from, and presumably the prevalence of tetanus in the county boroughs and the rural districts is clearly indicated. Whereas in males the mortality-rates for all the age-groups in urban districts are very similar to the corresponding rates in the country as a whole, the mortality in the aggregate of rural districts is considerably in excess of, and the mortality in county boroughs definitely less than, the average for the respective ages. A similar tendency is shown in the data for females.

SUMMARY AND CONCLUSIONS.

The present paper contains the results of an attempt to ascertain from data for the counties of England and Wales in the period 1901–09, the topographical variations that were present in the mortality from tetanus and the localities in which the concentration of the mortality was especially manifest, as well as to determine if any association could be traced between the local prevalence of tetanus and local features which might predispose to the disease.

It is clearly shown that tetanus was most prevalent in the south midland region of the country, in an area comprising the counties of Cambridgeshire, Berkshire, Hertfordshire, Buckinghamshire and Oxfordshire. These counties are alike in the respect that they overlie solid geological formations which are

TABLE III.—*Mean Annual Death-rates from Tetanus per Million at Certain Ages in the Two Periods* 1901–09 *and* 1919–25, *and the Death-rates at Ages in the Later Period Stated as Percentages of those in the Earlier Period.*

Age-groups.	Males. Mean annual death-rate per million. 1901–9.	1919–25.	Death-rate in 1919–25 as percentage of death-rate in 1901–09.	Number of deaths. 1901–09.	1919–25.	Females. Mean annual death-rate per million. 1901–09.	1919–25.	Death-rate in 1919–25 as percentage of death-rate in 1901–09.	No. of deaths. 1901–09.	1919–25.
	(1)	(2)	(3)	(4)	(5)	(1)	(2)	(3)	(4)	(5)
0–4	25·3	8·3	33	431	98	13·2	4·2	32	224	48
5–19	6·3	7·0	111	289	260	1·4	1·5	107	67	58
20–44	7·1	4·8	67	399	219	1·6	1·7	108	97	91
45–64	11·7	8·2	71	267	203	2·2	1·8	83	54	48
65+	8·5	5·7	67	59	39	2·9	1·6	56	25	15
All ages	9·7	6·5	67	1444	819	2·9	1·9	64	465	260

TABLE IV.—*Mean Annual Death-rates per Million from Tetanus at the Several Ages in England and Wales and its Sub-divisions in the Septennium* 1919–25.

	Males. 0–4	5–19	20–44	45–64	65+	All ages.	Females. 0–4	5–19	20–44	45–64	65+	All ages.
England and Wales	8·3	7·0	4·8	8·2	5·7	6·5	4·2	1·5	1·7	1·8	1·6	1·9
Rural districts	12·9	10·7	7·1	16·4	9·3	10·6	5·4	2·8	1·8	2·7	1·8	2·6
Urban districts	9·0	7·0	5·2	7·5	6·6	6·6	4·4	1·6	1·6	1·7	1·9	1·9
County boroughs	4·8	4·2	3·3	4·2	3·1	3·9	2·1	0·9	1·7	1·0	1·1	1·3
London	8·3	7·5	3·0	5·9	0	5·2	7·7	0·9	1·7	2·8	1·9	2·2

Death-rates at Ages from Tetanus in Divisions of Country as Percentages of Rates at Corresponding Ages in Country as a Whole.

England and Wales	100	100	100	100	100	100	100	100	100	100	100	100
Rural districts	155	153	149	199	165	165	129	180	106	150	108	136
Urban districts	108	101	108	91	116	102	105	105	96	97	117	102
County boroughs	58	61	69	51	55	60	49	56	100	58	68	70
London	99	107	62	72	...	80	184	59	103	154	113	114

for the most part cretaceous or oolitic in nature. The coincidence of a high mortality from tetanus and these formations is very interesting in view of Bullock and Cramer's experimental proof that calcium salts may be an adjuvant to the tetanus bacillus in producing the disease, but it cannot be proved that the high incidence is in any way determined by a possible excess of soluble calcium salts in the soil superficial to these strata. The more probable suggestion is that this region forms part of the chief arable area of England. Correlations computed between the regional tetanus mortality-rate and (1) the proportion of the male population engaged in agricultural pursuits, (2) the proportion of land under arable cultivation, (3) the number of horses per 1000 acres, and (4) the total number of cattle per 1000 acres respectively in the counties, indicate that there are definite direct associations between the tetanus mortality and the first two of these indices, no association with concentration of horses, and an inverse association with the density of the bovine population. The apparent negative association between the mortality and the concentration of cattle is, however, merely another expression of the relationship with the amount of arable land, as the concentration of cattle has a direct relation to the amount of permanent pasturage.

In the period under review, a very considerable proportion of the tetanus mortality occurred at ages under one year, and actually in the first three months of life. In males it is 26% and in females 44% of the total deaths at all ages. The proportion varies considerably in different counties, being in males as high as 62% and as low as 7% in certain instances. The regional mortality-rate from tetanus under one year has no association with the proportion of the population engaged in agricultural pursuits, nor the proportion of land under arable cultivation.

In recent years there has been a considerable decline in the mortality from tetanus, the average mortality-rate in 1919–25 at all ages being only approximately 65% of the average rate in 1901–09 in each sex. The decline at the earliest ages, has, however, been much more definite, as the mortality at ages 0–4 in the more recent period is only 33% of that in the earlier period. The decline in tetanus mortality has thus been in great part due to a decrease in tetanus neonatorum. This fact is in accordance with what Dr. Fildes has found in the statistics of the London Hospital.

I would like to express my indebtedness to Dr. Fildes for the interest he has shown and the encouragement he has given in the preparation of this paper.

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BLOOD-PHOSPHORUS IN HEALTH AND DISEASE: I.—THE DISTRIBUTION OF PHOSPHORUS IN HUMAN BLOOD IN HEALTH.

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It is proposed, in a short series of papers, to give an account of some quantitative investigations into the changes which take place in the phosphorus compounds of the blood in disease. To provide a satisfactory base-line, from which changes in disease may be measured, it has been found necessary to re-examine the phosphorus partition in normal human blood, and this communication will deal with the results of analysis of the blood in health; at the same time some aspects of our knowledge with regard to the several phosphorus-containing compounds which occur in blood, and their interrelationships, will be briefly reviewed.

A. FORMS IN WHICH PHOSPHORUS OCCURS IN HUMAN BLOOD.

Most of the work done prior to 1914 on the phosphorus compounds present in blood, and some of the work since that year, is invalidated because the lability of the equilibrium between the various forms of combination in which phosphorus is held in blood was not sufficiently realized. It is now well known that unless suitable precautions are taken, and particularly if the blood be laked and allowed to stand for a short time before precipitation of the proteins, fairly rapid changes ensue even at room temperatures in the inorganic phosphorus content of the blood, as the result of hydrolysis of phosphoric esters by enzymes liberated from the laked corpuscles (Martland, Hansman and Robinson, 1924; Lawaczeck, 1924). It is not so well known that even after precipitation of the proteins, blood filtrates which are allowed to stand in acid solution suffer slow hydrolysis of their organic phosphorus compounds. A typical example of this is given in Table I:

TABLE I.—*Effect of Standing in Acid Solution.*

Blood filtrate.	P in mg. per 100 c.c. blood. Hours after filtering off precipitated proteins.			
	0	5	24	48
Kept at room temperature 16–18°	4·89	5·06	5·62	5·82
,, 35°	4·89	...	6·50	7·10

Unlaked blood which is not allowed to become alkaline owing to loss of CO_2 may be kept for several minutes at room temperature before any detectable change in its inorganic phosphorus takes place. If the blood becomes more alkaline the inorganic phosphorus diminishes at first, then slowly rises again. If the blood is made slightly acid, the inorganic phosphorus rises from the start (Lawaczeck, 1924; Martland, Hansman and Robison, 1924). Owing to the loss of CO_2 on defibrination, with resulting alkalinity and synthesis of inorganic phosphate into an organic form, serum may contain less inorganic phosphorus than plasma. In assessing work on blood phosphorus it is therefore essential that the technique should be carefully considered, and the figures obtained compared with those derived from experiments which are known to have been carried out with the necessary precautions and controls. Laking the blood, or keeping it for some minutes before precipitating the proteins, allowing blood or blood-filtrates to stand long in acid or strongly alkaline solution or the use of high temperature for the protein coagulation destroy the quantitative significance of figures for the "inorganic," and for the so-called "organic acid-soluble" phosphorus of blood.

The phrase "acid-soluble phosphorus" brings us to the important work of Bloor (1918) on the phosphorus partition in normal human blood. He showed that the phosphorus compounds of human blood could be most conveniently divided into two classes:

(*a*) Acid-soluble phosphorus compounds, not precipitated when the proteins are removed by trichloro-acetic acid, and insoluble in alcohol-ether.

(*b*) Alcohol-ether-soluble phosphorus compounds precipitated by trichloro-acetic acid, and separable from class (*a*) by reason of their solubility in alcohol-ether.

Bloor found that these two classes were sharply separable from one another, and that their sum was equal, within experimental error, to the total blood phosphorus determined directly. Other forms of phosphorus (*e.g.* nucleic acid or phospho-proteins) are therefore not present in quantitatively significant amounts. Class (*a*) comprises inorganic phosphates and also a very much larger quantity of phosphorus in organic combination, from which it can be liberated by hydrolysis with acids (Bloor, 1918; Zucker and Gutman, 1923) or with enzymes (Kay and Robison, 1924; Kay, 1926[1]). The results of the last-named authors show that this fraction is composed of phosphoric esters. Class (*b*) comprises compounds of the type of lecithin. According to Bürger and Beumer (1913) the main ether-soluble phosphorus compounds of blood are kephalin and sphingomyelin; lecithin itself is present in relatively small amounts only.*

Actually the compounds in the two classes above do not comprise the whole of the phosphorus compounds of blood. There is present also a relatively very small amount of nucleic acid, contained in the leucocytes, which does not appear in either (*a*) or (*b*) above. It is so small that it cannot be shown to be present in whole blood by direct methods of analysis, but may be demonstrated by separation and analysis of the leucocytes. Phosphorus in

* Until this finding is confirmed or disproved, it is best to record this fraction as "lipin P." It is clearly inadvisable to multiply the ether-alcohol-soluble P by 25 and call it lecithin as is sometimes rather loosely, done.

organic combination is also present in specimens of serum albumin and globulin which have not been extracted with alcohol-ether, but according to Sørensen (1926) this organic phosphorus is probably *not* an integral portion of the protein molecule, since alcohol-ether extraction at $-4°$ removes it almost completely. He believes that both these proteins, when pure, will be found to be phosphorus-free.

Although it has been known for several years (*cf.* Panella, 1902, 1903) and re-discovered a number of times that organically bound phosphorus, other than nucleoprotein and lipin phosphorus, is present in considerable quantities in blood, it was not till recently that Jackson (1923, 1924) isolated and identified one of these compounds as being adenine nucleotide. The next contribution was made by Kay and Robison (1924), who showed that there were at least two classes of acid-soluble organic phosphorus compounds in blood, some 25–35% of the acid-soluble phosphorus being hydrolysed rapidly at 38° by means of an enzyme which could be extracted from bone, whilst the remainder of the organic phosphorus was not hydrolyzed by this agent. Adenine nucleotide would belong to the hydrolysable fraction. Since then Goodwin and Robison (1924) have separated a reducing phosphoric ester (possibly a derivative of a hexose phosphoric ester), and Greenwald (1925) a compound without reducing properties which he has shown to be a salt of the diphosphoric ester of *l*-glyceric acid. It is convenient at this stage to bring together in one table all the phosphorus compounds for whose existence in blood there is definite evidence (Table II):

TABLE II.—*Forms in which Phosphorus Occurs in Human Blood.*

Acid-soluble phosphorus	1. Inorganic phosphate.	
	2. Nucleotide	Adenine nucleotide, possibly also a pyrimidine nucleotide (Jackson, 1924).
	3. Phosphoglyceric acid.	
	4. Reducing phosphoric ester.	
	5. At least one other phosphoric ester.	
	2, 3, 4 and 5 form the "ester phosphorus" of blood, by which name this fraction will be described in this paper.	
Lipin phosphorus	6. Lecithin.	
	7. Kephalin.	
	8. Sphingomyelin.	
Residual phosphorus (very small in total quantity)	9. Nucleic acid.	

B. CELLS AND PLASMA.

Blood is a system containing several separable elements, and these—plasma, red cells, leucocytes and platelets—contain very different amounts of the various phosphorus compounds just mentioned. The leucocytes and platelets are considerably richer in phosphorus compounds than the red cells

(*cf.* a few figures given by Kay, 1926[2]). Leucocytes, however, are present in normal blood in such relatively small amounts that their contribution to the analytical figures in any one determination on whole blood is probably within the experimental error. This is *not* so for the mean value of a series of determinations, and when dealing with the values for the red cells such as the "phosphorus index" described later, a small correction should, strictly, be made. Certainly in abnormal bloods, before the analysis can be accepted, the quantity of leucocytes must be checked, either by observing their volume in the high-speed hæmatocrit, or by counting.

Practically all, but not quite all the ester phosphorus of blood is present in the cells. It has been demonstrated by Martland and Robison (1926), and by Kay (1926[2]), that ester phosphorus compounds are also present in normal human plasma, and that they are hydrolysed by tissue phosphatases, but the quantity of phosphorus so held by the plasma is only about 1% of the total ester phosphorus in 100 c.c. of whole blood. Of the remaining 99%, 97% is present in the red cells, and about 2% in the leucocytes and platelets. Platelets from human blood have not yet been examined, but the observation mentioned above for guinea-pig platelets has since been confirmed on two specimens of platelets from horse blood. The phosphorus content of horse platelets is high—considerably higher than that of red cells—and of the same order as that of leucocytes, and the same may be assumed with some degree of safety to hold for human platelets until analytical figures are available.

C. ANALYTICAL METHODS FOR NORMAL BLOOD.

It is desirable, for reasons mentioned earlier, that the analytical methods we have used should be described in some detail.

For the complete analysis 22–25 c.c. of blood is taken from an arm vein into a dry syringe, and 0·5 c.c. is introduced into a small tube containing the necessary quantity of heparin to prevent coagulation. This portion is used for the hæmatocrit determination of the percentage volume of red cells, and in some cases for a cell-count also. The remainder of the blood is poured on to 30 mgm. of finely powdered potassium oxalate in a large test-tube, which is closed and inverted two or three times. From this tube about 1·5 c.c. of blood is placed in a smaller tube for total phosphorus determination, and for red-cell count. Exactly 7 c.c. is run into 52 c.c. of water in a tall, accurately graduated measuring cylinder. The liquid in the cylinder is carefully made up to 70 c.c. with 25% trichloro-acetic acid and shaken hard for a few seconds. The final trichloro-acetic acid concentration is thus 3·9%, or approximately $\frac{N}{4}$. After standing ten minutes, the whole is filtered, and 10 c.c. of filtrate taken at once for a determination of free phosphate by Briggs's method. 5 c.c. of filtrate in triplicate is run into each of three Pyrex boiling tubes for a determination of the total (inorganic + ester) phosphorus, and the remaining filtrate (some 25 c.c.), measured exactly, is brought to pH 8·9 with 10% NaOH, using phenolphthalein as indicator. Meanwhile, 2 c.c. of original whole blood has been pipetted into 35 c.c. of ether-alcohol mixture in a 50 c.c. flask for determination of lipin phosphorus by the method of Randles &

Knudson (1922). The remainder of the blood (9–10 c.c.) is rapidly centrifuged, the plasma separated, measured and run into 15 c.c. of water in a 25 c.c. flask, 3·5 c.c. of 25% trichloro-acetic acid added, and water up to the mark. After inverting several times and keeping for 10 minutes, the contents of the flask are filtered, $\frac{1}{3}$ of the filtrate taken for free P and $\frac{1}{3}$ run into each of two Pyrex boiling tubes for duplicate determinations of total (inorganic + ester) P of the plasma.

The filtrate from whole blood (at pH 8·9) is measured, and 5 c.c. pipetted into each of 4 test-tubes, containing 5 c.c. of Sørensen glycine buffer (of pH 8·9 at 38°) and 0·2 c.c. of chloroform. To each tube 0·5 c.c. of extract of pig's kidney containing phosphatase (Kay, 1926[1], p. 804) is now added, and immediately, to two of the tubes, 2 c.c. of 25% trichloro-acetic acid to inactivate the enzyme. These tubes are placed in the cold box. The other two tubes are now incubated for 18 hours in a water-bath at 38°, cooled, and 2 c.c. acid added to each. Control tubes containing enzyme and buffer alone, incubated and not incubated, are also used. The contents of the tubes are filtered, and 10 c.c. of each filtrate taken for a colorimetric phosphorus determination. The amount of hydrolysis in 18 hours at 38° represents the enzyme-hydrolysable P. Of the 1·5 c.c. of oxalated blood placed aside at the beginning, 0·3 c.c. is measured in a long pipette into 4 c.c. of water in each of 3 Pyrex boiling tubes, for total phosphorus determination.

To all the tubes for total P determinations 1 c.c. of a mixture of nitric acid 2 parts, sulphuric acid 1 part by volume is added, and the contents of the tube carefully evaporated down over wire gauze over a small flame, or in a small electric heater like that recommended by Stanford and Wheatley (1925), until either charring occurs, or white fumes appear without charring. In the former case 2 or 3 drops of pure nitric acid are added, and the tube reheated till white fumes appear, when the tube is allowed to cool, and 2 drops of Merck's perhydrol added. In the latter case 2 drops perhydrol are added at once. The tubes are again carefully heated until white fumes begin to appear, cooled, about 8 c.c. water added, boiled for 10 minutes and left overnight. Next morning the contents of each tube are quantitatively transferred to a 25 c.c. flask, and the analysis completed by the Briggs (1922) technique, using ammonium molybdate instead of sulphuric acid-molybdate mixture, since the correct amount of sulphuric acid is already in the flasks.

D. COMMENTARY UPON THIS TECHNIQUE.

Anti-coagulant.—Briggs (1922) stated that the amount of oxalate required to prevent coagulation of blood did not interfere with his method of inorganic phosphorus determination. This we can confirm. Owing to the relatively high osmotic pressure of potassium oxalate, it is not possible to use this anti-coagulant for determining the relative corpuscular volume. Heparin is therefore used for the hæmatocrit blood, since the osmotic pressure of this substance in the amounts required to prevent clotting (1 mgm. for 5 c.c. blood) may be neglected. It cannot, however, be used when it is desired to perform an organic phosphorus determination on the blood, since it contains, as was pointed out to one of us by Dr. R. Robison, a high proportion of organically

bound phosphorus. We have found in two batches of heparin (Dunning) that the organic phosphorus is between 4 and 5%, and that it is, at least in part, removed as inorganic phosphate by the action of kidney phosphatase.

Inorganic phosphate.—The changes which follow in the inorganic phosphorus content of blood after laking, though fairly rapid, particularly of course at 38°, are far from instantaneous. Thus it is possible to lake blood and precipitate the proteins at room temperature with trichloro-acetic acid as we have described without any appreciable increase in the inorganic phosphate content, even if the blood be allowed to stand for 1 or 2 minutes after laking. (See Table III).

TABLE III.—*Changes in Inorganic P of Blood after Laking.*

Mg. P per 100 c.c. blood.

Blood specimen.		Time of addition of trichloroacetic acid after laking (minutes). 0*	0·5	1	3	5	10
Normal blood	1	2·77	2·76	...	2·75	...	...
,, ,,	2	2·9	2·7	2·8	2·9	2·9	...
,, ,,	3	...	...	2·42	2·36	2·42	2·5
,, ,,	4	2·92	...	...	2·95	2·98	3·01
Pathological blood	1	...	...	3·26	3·35	...	3·42
,, ,,	2	3·6	...	...	...	3·9	...
,, ,,	3	6·3	...	...	...	...	6·6
,, ,,	4	4·29	...	4·24	4·36	4·42	...

* Method of precipitation of Fiske and Subbarrow (1925).

The inorganic phosphorus determination is not infrequently interfered with by a slight opalescence, which develops on the addition of the molybdic acid reagent to the filtrate after precipitation of the blood colloids with trichloro-acetic acid. Fiske and Subbarrow (1925) suggest the use of stronger, *i. e.* 8%, trichloro-acetic acid to be quite sure of obtaining a protein-free filtrate. Even this method does not, in our experience, invariably avoid the opalescence, which is probably not entirely due to proteins or their hydrolysis products. In our earlier experiments, a drop of capryl alcohol was added to the 52 c.c. of distilled water in the measuring cylinder just described, to prevent frothing and to increase the rate of filtration. When this was omitted, addition of molybdic acid to the filtrate gave less, or no cloudiness.

Total phosphorus.—After oxidizing the organic matter of blood with sulphuric-nitric acid and hydrogen peroxide, the residue is boiled with water for ten minutes and *left overnight* before making the determination. When known amounts (0·1 mgm.) of inorganic phosphate are treated in this way, we find that if the phosphorus is determined *immediately* after cooling it is 1 to 3% too low. On standing overnight the apparent amount of phosphorus rises by 1% or more, giving a figure closer to the theoretical. This small rise may be due either to hydrolysis of some complex acid, *e. g.* phosphoryl-sulphuric acid or pyrophosphoric acid, or to liberation of phosphoric acid taken up by the glass (*cf.* Martland and Robison, 1924).

Presence of iron.—Fiske and Subbarrow (1925) showed that relatively large amounts of iron introduced a serious error in the Briggs method for total P determination. This is not the case with the small amount of iron present in human blood when P is determined by the method just described. Although the percentage of total Fe in normal human blood is slightly greater than the percentage of total P, the amount of iron in 0·3 c.c. blood (0·18 mgm.) added as $FeCl_3$ to a standard phosphate solution containing 0·100 mgm. P does not affect the readings by more than 1%.

In another experiment, 0·3 c.c. of blood was accurately pipetted into each of 9 Pyrex tubes. To 3 of these tubes was then added 0·18 mgm. Fe as $FeCl_3$; to 3, 0·09 mgm. Fe; to the remaining 3 nothing was added. All were then incinerated in the usual way with H_2SO_4 and HNO_3, followed by H_2O_2, and the P determination made as usual. (Results in Table IV).

TABLE IV.

Iron present.	Colorimetric readings (average of triplicate combustions). Standard P.	Total blood P.
Amount in 0·3 c.c. blood + 0·18 mgm.	0·100 mgm. at 30	29·0
" " " + 0·09 "	" "	29·1
" " "	" "	29·0

Iron added to whole blood before combustion, even in the same amount as that present in the blood itself, does not therefore influence colour production.

E. ANALYTICAL RESULTS.

The results of analysis of the blood of 31 men and women, all normal subjects, are given in Tables V–VIII. They have been divided into two groups, depending on whether the blood was taken (*a*) after an overnight fast, or (*b*) within 1 to 3 hours after breakfast. The highest, lowest and mean values are shown at the foot of each table. The subjects were, in the majority of cases, laboratory or hospital workers. A few convalescents from mild disorders have been included.

The analytical findings may be summarized as follows:

(*a*) The distribution of phosphorus in venous blood of normal adults is fairly constant from one individual to another.

(*b*) There are slight differences due to sex. Thus the mean ester phosphorus is greater in men than in women, and the portion of this which is hydrolysable by phosphatase is less.

(*c*) The variations between the sexes, and from one individual to another in the ester phosphorus, may be correlated with differences in the relative volume of the red corpuscles to the plasma.

(*d*) There are slight variations due to alimentary conditions. These are shown separately in Table IX, where figures for men's blood and women's blood *before*, are compared with those *after* a meal (breakfast). After a meal there is a slight diminution in inorganic P, a diminution in phosphoric ester hydrolysable by phosphatase, and a corresponding increase in the ester fraction resistant to enzyme hydrolysis.

(*e*) There is possibly a slight rise in lipin-phosphorus after an ordinary meal.

TABLE V.*—*Normal Men Fasted Overnight.*

P in mgm. per 100 c.c. blood.

TABLE V.*—*Normal Men Fasted Overnight.*

No. of experiment.	Age of subject.	P in mgm. per 100 c.c. blood. Free.	Total acid-soluble.	Ester.	Enzyme hydro-lysable.	Non-hydro-lysable.	Lipin.	Total (by addition).	Hæmatocrit % red corpuscles.	Organic P index.	Remarks.
1	33	3·2	27·5	24·3	6·0	18·3	11·2	38·7	46	53	...
2	33	3·4	27·1	23·7	5·4	18·3	11·3	38·4	44	54	Same individual as 1, a fortnight later.
3	32	3·0	28·0	25·0	7·4	17·6	12·7	40·7	44	57	...
4	23	2·8	24·4	21·6	5·9	15·7	12·0	36·4	41	53	...
10	48	3·1	30·6	27·5	8·2	19·3	12·3	42·9	51	54	...
18	25	3·0	24·3	21·3	7·8	13·5	11·9	36·2	44·5	48	...
19	24	3·2	25·5	22·3	7·0	15·3	11·3	36·8	42·5	52·5	...
20	26	3·2	25·3	22·1	7·2	14·9	13·4	38·7	44	50	...
Highest		3·4	30·6	27·5	8·2	19·3	13·4	42·9	51	57	
Lowest		2·8	24·3	21·3	5·4	13·5	11·2	36·2	41	48	
Mean		3·1	26·6	23·5	6·9	16·6	12·0	38·6	44·6	52·7	

TABLE VI.—*Normal Men after Breakfast.*

No. of experiment.	Age of subject.	P in mgm. per 100 c.c. blood. Free.	Total acid-soluble.	Ester.	Enzyme hydro-lysable.	Non-hydro-lysable.	Lipin.	Total (by addition).	Hæmatocrit % red corpuscles.	Organic P index.	Remarks.
7	32	2·6	25·8	23·2	6·1	17·1	11·5	37·3	43·5	53	...
8	30	3·3	27·7	24·4	7·3	16·6	12·9	40·6	46	53	...
9	19	3·5	27·9	24·4	6·4	18·0	12·9	40·8	43	57	...
11	33	2·6	27·4	24·8	6·4	18·4	11·9	39·3	44	56	Same individual as 7, eight months later.
12	30	3·0	28·6	25·6	7·0	18·6	10·8	39·4	43	52	...
14	25	2·6	25·0	22·4	5·8	16·6	11·4	36·4	43	52	...
15	25	3·0	23·5	20·5	6·0	14·5	11·1	34·6	38	54	...
17	26	2·4	25·9	23·5	5·0	18·5	12·1	38·0	43·5	54	...
21	20	3·0	27·0	24·0	5·5	18·5	12·6	39 6	44·5	54	...
Highest.		3·5	28·6	25·6	7·3	18·6	12·9	40·8	48·5	57	
Lowest.		2·4	23·5	20·5	5·0	14·5	10·8	34·6	38·0	52	
Mean		2·88	26·5	23·6	6·16	17·4	11·9	38·4	43·7	54·0	

* Our figures are given in terms of P. By different authors phosphorus analyses are variously given in terms of P, P_2O_5 or H_3PO_4. It would much facilitate comparison if there were a general agreement to present such analyses in one of these terms; the most logical seems to the present authors to be P. In any case, there seems little excuse for presenting phosphorus analyses in terms of the antiquated P_2O_5. Nitrogen analyses are not presented as N_2O_5.

TABLE VII.—*Normal Women Fasted Overnight.*

No. of experiment.	Age of subject.	P in mgm. per 100 c.c. blood. Free.	Total acid-soluble.	Ester.	Enzyme hydro-lysable.	Non-hydro-lysable.	Lipin.	Total (by addition).	Total (by combustion).	Hæmatocrit.	Organic P index.
5	37	3·0	24·6	21·6	9·4	12·2	Lost	...	...	39·0	55
6	30	3·1	23·4	20·3	7·7	12·6	12·8	36·2	...	37·5	54
29	23	3·4	25·9	22·5	6·1	16·4	10·5	36·4	35·6	43·5	52
30	53	3·5	24·0	20·5	7·3	12·9	12·8	36·8	37·4	41·0	50
31	58	2·5	26·7	24·2	8·1	16·1	13·1	39·8	39·6	47	51·5
33	...	3·8	24·1	20·3	5·9	14·4	11·1	35·2	35·9	36·5	55
34	...	3·5	25·4	22·1	6·1	16·0	10·4	35·8	36·3	43·5	51
35	22	2·8	25·2	22·4	6·7	15·7	10·8	36·0	36·6	41·5	54
Highest		3·8	26·7	24·2	9·4	16·4	13·1	39·8	39·6	47	55
Lowest		2·5	23·4	20·3	6·1	12·2	10·4	35·2	35·6	36·5	50
Mean		3·2	24·9	21·7	7·2	14·5	11·6	36·6 (av. of 7)	36·9 (av. of 6)	41·2	52·8

TABLE VIII.—*Normal Women after Breakfast.*

No. of experiment.	Age of subject.	P in mgm. per 100 c.c. blood. Free.	Total acid-soluble.	Ester.	Enzyme hydro-lysable.	Non-hydro-lysable.	Lipin.	Total (by addition).	Total (by combustion).	Hæmatocrit.	Organic P index.
13	24	3·3	22·9	19·6	5·6	14·0	11·8	34·7	...	41	48
16	25	3·2	25·6	22·4	6·7	15·7	13·8	39·4	...	38	59
22	26	2·8	25·7	22·9	6·6	16·3	Lost	...	...	42	55
24	26	2·8	23·5	20·7	6·5	14·2	Lost	...	34·3	39·5	52
25	25	3·0	23·9	20·9	5·9	15·0	12·6	36·5	37·0	42·0	50
27	45	3·1	27·4	24·3	7·7	16·6	11·3	38·7	39·3	45·5	53·5
28	30	2·7	23·0	20·3	6·6	13·7	12·0	35·0	35·7	42·0	48·0
32	40	2·6	27·2	24·6	7·8	16·8	12·9	40·1	41·0	44	56
Highest.		3·3	27·4	24·6	7·7	16·8	13.8	40·1	41·0	45·5	59
Lowest		2·6	22·9	19·6	5·6	13·7	11·3	34·7	34·3	38	48
Mean		2·94	24·9	22·0	6·68	15·3	12·4	37·4 (av. of 6)	37·5 (av. of 5)	41·8	52·6

TABLE IX.—*Effect of a Meal on Blood-phosphorus.*

	P in mgm. per 100 c.c. blood.									
	Free.	Total acid-soluble.	Ester.	Hydro-lysable.	Non-hydro-lysable.	Lipin.	Total.	Hæma-tocrit.	*Organic P index.	Hydro-lysable P index.	Non-hydro-lysable P index.
Men + women before meal	3·15	25·7	22·6	7·05	15·55	11·8	37·6	42·9	52·8	16·4	36·1
Men + women after meal	2·9	25·6	22·9	6·42	16·4	12·2	37·9	42·8	53·3	15·0	38·3

* Phosphorus index is described below.

Although total phosphorus determinations by the direct method have not been done on all samples of blood, the average values agree very well with those obtained by adding together the total acid-soluble phosphorus and the lipin phosphorus, and support Bloor's statement that forms of phosphorus other than acid-soluble and lipin phosphorus occur in normal blood in quite unimportant amounts.

The direct and "added" totals do not always agree in individual cases. It must, however, be pointed out that the latter total is the sum of two figures each obtained by combustion, and the disagreement rarely exceeds 2%. Somewhat greater discrepancies are met with in pathological bloods—this point will be dealt with in a later communication.

F. THE "PHOSPHORUS INDEX."

The analytical figures show a definite correlation between the fraction of the volume of the blood occupied by the red cells, as determined by the hæmatocrit, and the total amount of phosphorus present in whole blood. If the latter value be divided by the former (which can probably be determined more accurately than the red-cell count, and is independent of the size of the individual cells), a figure is obtained which is remarkably constant in health, and even in many diseases found to be little changed. This constant we have called the "phosphorus index" of the blood. It represents the amount of ester phosphorus in milligrammes present in 100 c.c. of red cells. It has practically the same value (53) for men as for women, and, according to our findings, very rarely varies *in health* more than 10% on each side of the mean. The phosphorus index appears to be one of the most stable blood constants* of the normal organism—more stable, for example, than the hydrogen ion concentration.

In calculating this value it would be more strictly correct, before dividing by the hæmatocrit value, to subtract from the total amount of ester phosphorus in blood the small amount in the plasma and in the leucocytes. This has not been done, however, for two reasons: (*a*) to determine the very small amount of ester phosphorus in the plasma or in the leucocytes in any one case with accuracy requires relatively large amounts of blood; (*b*) the amount is so small

* Myers (1924, Table IV) gives the normal range of variation of the (acid-soluble) organic phosphorus of the corpuscles as 40–75 mg. P per 100 c.c. This range is, according to our findings, far too wide. It is, however, interesting to note that his average figure (53) for the number of mgm. of (acid-soluble) organic P in 100 c.c. blood, which corresponds with our phosphorus index, is in exact agreement numerically with our average phosphorus index.

that its numerical effect in diminishing the phosphorus index, calculated as originally described, is less than two units for the blood of normal individuals. Thus we have found an average value of only 0·3 mgm. P per 100 c.c. plasma for the ester phosphorus in the plasma of ten normal individuals, there being apparently a rather larger amount in fasting plasma than in plasma after a meal. It has been shown that most of this organic phosphorus is enzyme-hydrolyzable (Kay, 1926[2]). The low figure of 0·3 mgm. P % is not necessarily true for the plasma in disease.

It is also of interest to calculate the "index" for the enzyme-hydrolyzable and non-hydrolyzable fractions of the ester phosphorus. Table X shows the average values for men's and women's blood before and after a meal.

TABLE X.

	Phosphorus index.	Enzyme-hydrolyzable index.	Non-hydrolyzable index.
Normal men fasting	52·7	15·3	37·3
„ „ 1–3 hours after food	54·0	14·1	39·9
Normal women fasting	52·8	17·5	35·2
„ „ 1–3 hours after food	52·6	16·0	36·6

G. RESULTS OF OTHER WORKERS.

The results of other workers and our own are shown side by side in Table XI. Bloor's (1918) figures, determined nine years ago by an entirely different

TABLE XI.

P in mgm. per 100 c.c. blood.

Number of individuals.	Free.	Total acid-soluble.	Ester.	Lipin.	Total.	Percentage corpuscles.	Organic P index.
Present Authors.							
15 men	3·0	26·6	23·6	12·0	38·5	44·0	53
16 women	3·1	24·9	21·8	12·0	37·0	41·5	53
Bloor.							
16 men	4·1	27·9	23·8	11·8	40·2	44·0	54*
10 women	4·1	26·8	22·7	12·0	39·3	41·5	55*
Buckman, Minot, Daland and Weld.							
6 normals	3·4	...	...	...	37·7	44·3	...
Stanford and Wheatley.							
5 normals	3·0	26·9†	23·9	10·7	36·8	46·3	51

* Calculated by us from the authors' figures.
† Obtained by adding "inorganic" and "ester P" together.

analytical technique, are in very fair agreement with ours.* There is a discrepancy between his figures and ours for inorganic phosphorus and for total phosphorus. A possible reason for the higher figure of Bloor for inorganic phosphate is that he allowed the corpuscles to stand, laked, for ten minutes before precipitating the proteins, which in the light of more recent experience would undoubtedly tend towards an increase in the inorganic at the expense of the ester P. Buckman, Minot, Daland and Weld (1924) obtained values for the inorganic and total phosphorus and the percentage of corpuscles in six normal bloods which agree well with our averages for these three determinations. Stanford and Wheatley (1925) analysed the blood of five individuals, and, except for a rather lower lipin phosphorus, obtained figures agreeing fairly well with our own.

A large number of inorganic phosphate figures for normal human blood have appeared during the past few years; those of Bell and Doisy (1921), Briggs (1923), Jones and Nye (1921), Tolstoi (1923), de Wesselow (1922), McKellips, de Young and Bloor (1921) and Havard and Reay (1925) may be mentioned, and with the exception of Roller (1926) all agree to an average inorganic phosphate value of some 3·0 mgm. P per 100 c.c. Roller's higher figure (average 4·7) may possibly be ascribed to his leaving the blood filtrate overnight in hydrochloric acid solution. The slight fall in the inorganic P of blood after a meal which our figures show may be related to the secretion of inorganic phosphate into the upper end of the intestine which Bergeim (1926) has shown to take place during digestion.

H. ESTER PHOSPHORUS AN INTEGRAL CONSTITUENT OF THE RED CELL.

One of the most striking results brought out by our analyses is the constancy of the ester phosphorus of the red cells in health. Per unit volume of red-cell material, there is a constant amount of ester phosphorus, which, like the hæmoglobin, remains firmly attached to the cell until the latter is lysed. The compounds in blood may be considered as belonging to two classes: (*a*) those which form a portion of the permanent furniture of the blood as a tissue, and (*b*) those which are being transported by the blood from one organ to another. As far as the phosphorus compounds of normal blood are concerned, the ester phosphorus of the cells is mainly, if not entirely, (*a*). The ester phosphorus of plasma is probably mainly (*b*), whilst the inorganic phosphorus and the lecithin phosphorus of plasma are partly (*a*) and partly (*b*).

Possible Osmotic Effects of the Ester Phosphorus of the Corpuscles.

It has been shown by one of us (Kay, 1926[2]) that when the corpuscles are laked, the ester phosphorus compounds are readily dialyzable at 0° through membranes which keep back the blood colloids. By what mechanism the relatively large amount of apparently diffusible organic phosphorus is maintained practically exclusively within the corpuscles is as yet unknown. Though

* Bloor's figures appear in the original paper as H_3PO_4 per 100 c.c. of plasma or 100 c.c. red cells. In recalculating his figures, we have assumed that his men had an average of 44, and his women an average of 41·5 % corpuscles in their blood.

their diffusibility through the red cell membrane is limited by its peculiar semi-permeability, they must be osmotically active. All the known phosphoric esters of natural occurrence are dissociated in solution into at least two, and usually three, ions per atom of P, so that the assumption that the ester phosphorus compounds of the corpuscles are dissociated into two ions is a conservative one. We have shown the concentration of ester P to be 0·53 gm. per litre of corpuscles; thus the solution within each corpuscle is $\frac{0{\cdot}53}{31}$ normal for P. The corpuscles contain, however, at least 33% of their weight as hæmoglobin, so that the actual normality of the ester phosphorus in terms of the water present is more nearly $\frac{0{\cdot}53}{31} \times \frac{100}{67}$ N.* Assuming that the ester phosphorus compounds of the corpuscles are completely dissociated into two ions, this normality would correspond with a Δ for these compounds of 0·097° or between $\frac{1}{5}$ and $\frac{1}{6}$ of the Δ of the blood (0·55°). The osmotic activity of the phosphoric esters of the cells, functioning, as they appear to do, at the hydrogen ion concentration of the blood, as anions, together with the base held by them, is thus not less than $\frac{1}{6}$ of that of the whole corpuscular contents. That this consideration is not entirely an academic one it is hoped to show in a later paper.

SUMMARY.

The partition of phosphorus in the blood in 31 normal individuals has been determined, and the analytical methods used critically discussed. The constancy of the ester phosphorus of the red cells in health has been demonstrated, and certain considerations arising out of the analytical data are presented.

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* It will be observed that no notice has been taken in this calculation of the other solid constituents of the corpuscles, which would still further decrease the amount of water in which the diffusible esters are dissolved.

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THE SPECIFICITY OF THE ROUS SARCOMA VIRUS AS TESTED BY EXPERIMENTAL EMBRYOMA IN THE CHICKEN.

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WHEN embryo tissues are introduced into a fully-developed host of the same species they will grow for a time, but invariably retrogress and finally disappear. The success or failure of a graft of this nature depends on many different factors, *e. g.* age of embryo, age of host, general health of host, pregnancy, etc. Many attempts have been made to stimulate these embryo grafts to continued growth, with but little success.

Object of present experiments.—A cell-free filtrate of Rous sarcoma when injected into the breast of a chicken appears to produce a tumour by its action on the connective tissue alone, the muscle being invaded and destroyed secondarily. In the following experiment an attempt was made to induce the filtrate to affect embryonic cells, *i. e.* cells other than fibroblasts. Embryonic tissue seemed most suitable for this purpose, owing, firstly, to its active proliferation, secondly, to its short time of survival when implanted into the tissues of a more fully-developed host.

An attempt was also made to produce a pigmented tumour by means of embryonic retinal cells. Theoretically, if these cells were affected by the

active agent of the sarcoma filtrate, a pigmented tumour should develop, the cells of which could readily be distinguished from the connective tissue of the host, or from other cells entering into the composition of the tumour.

TECHNIQUE.

Embryos.—The chick embryos used were from 7 to 14 days old, and were from eggs of the same breed as the inoculated birds. No attempt was made to keep to the same strain. The inoculated birds were approximately eight weeks old. The embryos were minced finely with sharp scissors, separated from excess tissue juice by centrifuging and inoculated intramuscularly into the breast of the host. Approximately half an embryo was used in each case—0·5 c.c.

Filtrate.—Clear filtrate of Rous sarcoma was made by grinding the tumour with sand and adding saline in the proportion 2·5 gm. tumour to 100 c.c. saline. The mixture, after allowing the grosser particles to settle, was filtered through packed paper pulp and sand according to the method described by Gye (1925). Embryo mince, suspended in the filtrate for half an hour at 37° C., was repeatedly washed in Ringer solution to free it from excess filtrate; it was then mixed with a small quantity of embryo tissue juice to enable it to be taken up by a syringe, and inoculated intramuscularly.

Embryo retinal cells.—The vitreous and lens of the eye were withdrawn through a posterior incision, leaving the retina exposed on the inner surface of the sclera, and from this it was separated and cut into fragments.

EXPERIMENTAL RESULTS.

1. *Effect of Inoculation of Embryo Mince.*

0·5 c.c. (approximately half an embryo) was inoculated into the breast-muscle of a young chicken, with the following results:

(*a*) A firm nodular tumour developed which rapidly enlarged for 7 to 10 days, then as rapidly retrogressed, leaving little or no trace after 2 to 3 weeks. On histological examination the tumour was found to consist of rounded nodules of cartilage and small cysts lined by columnar or squamous epithelium embedded in connective tissue.

(*b*) If the tumour was removed after 7 days and reinoculated into another bird no further development occurred.

2. *Effect of Inoculation of Embryo Mince Treated with Filtrate.*

Into the right breast of each bird approximately 0·5 c.c. of embryo mince treated with filtrate was inoculated intramuscularly, with the following results:

(*a*) In the right breast a rapidly growing tumour developed, which soon occupied the whole breast-muscle (28 days). Histologically, the tumour appeared as a spindle-cell sarcoma invading muscle, and contained islets of embryonic tissue embedded in it, *e.g.* small masses of cartilage, epithelium

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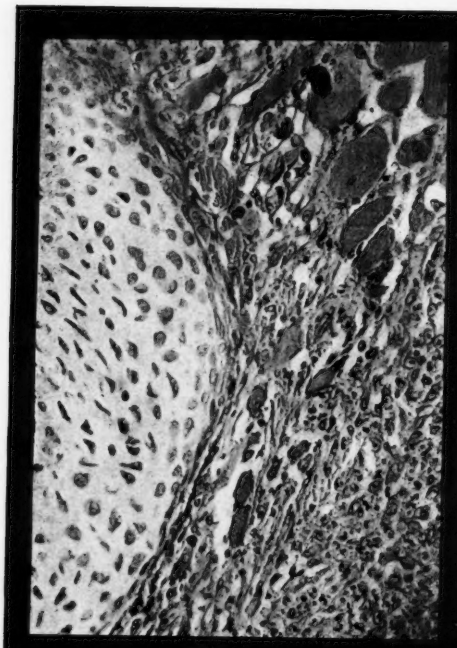

FIG. 1.

FIG. 2.

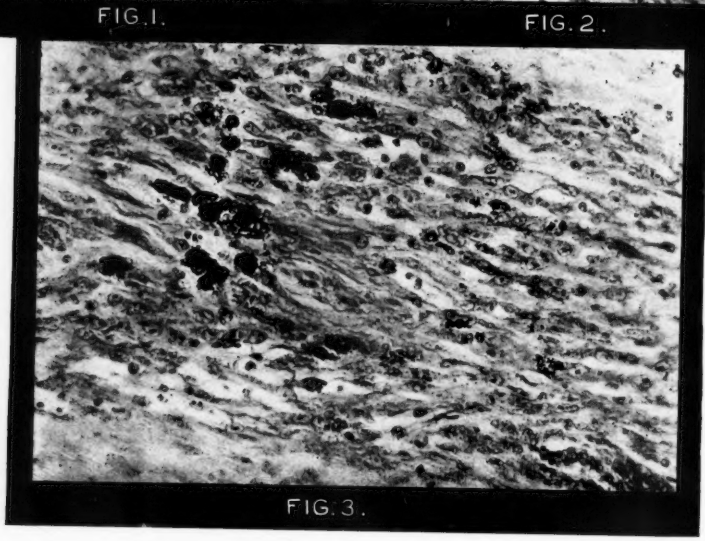

FIG. 3.

lining cysts, and small nests of keratinizing squamous epithelium (Figs. 1 and 2).

(*b*) Embryonic structures could be demonstrated in these tumours up to 4 weeks—a contrast to the more rapid retrogression that occurs in a simple embryonic implant.

(*c*) If the mixed tumour was removed after 7 days and reinoculated into another bird, a sarcoma developed which contained no recognizable embryonic elements.

As a control there was injected into the left breast an equal quantity of embryo mince which had been incubated in Ringer solution for half an hour at 37°C. This resulted in the development of a small nodular tumour, which retrogressed if left *in situ*, and was not transmissible by reinoculation into another bird.

3. *Effect of Inoculation of Embryonic Retinal Cells Treated with Filtrate.*

Into the right breast of each bird the retinal cells which had been incubated with filtrate were inoculated, and into the left breast an equal quantity of retinal tissue which had been incubated with saline. The amount of tissue introduced was small—the retina from two eyes constituting one dose.

Result.—(1) A palpable tumour developed within 7 days in the right breast. Histologically, the tumour consisted of a spindle-cell sarcoma showing invasion of muscle. Small collections of pigment were present between the tumour-cells, some of which contained pigment-granules in their cytoplasm (Fig. 3).

(2) Reinoculation of this tumour produced a sarcoma with no pigmentation.

(3) The control side, left breast, showed no demonstrable growth. Minute pigmented spots were noticed in one case.

DISCUSSION.

In previous work on the behaviour of mixed tumour and embryo implants mammalian tumours have been employed. Rous (1910) noted the transient development of embryo tissue in mice. He observed that successful grafts evoked a stroma response on the part of the host, and that this essential "stroma reaction" and vascularization was similar to that produced by successful tumour grafts, as had been shown to be the case with transplantable tumours by Bashford (1905), the only difference being that whereas the tumour grafts continued to grow, the embryo grafts soon disappeared. Immunity to embryo grafts could be produced by methods similar to the production of tumour immunity—the immunity apparently depending in each case on the absence of the "stroma reaction."

Rous (1911) also found that the resulting growth from grafts composed of both tumour and embryo cells depended on the balance of "avidity" of the two tissues, thereby confirming the work of Apolant on mixed sarcoma and carcinoma grafts. Thus a rapidly growing tumour will soon outgrow the embryonic element, whereas embryo tissue outgrows a delicate tumour, which survives as small islands of tumour tissue embedded in the embryo mass. In more balanced grafts where the stroma is well developed, the two tissues grow side by side, and in some instances he has described definite union occurring

between the tumour-cells (mouse carcinoma) and the squamous epithelium of the embryonic element. Fischera (quoted by Rous) in a similar experiment with rats, showed that rat embryo and rat sarcoma developed side by side for a time, but that the embryonic elements eventually retrogressed, leaving only the sarcoma.

The structure, development and differentiation of embryonic chicken tissues, both *in vivo* and *in vitro*, has been described in detail by Strangeways (1926).

Much work on this subject has been described by Carrel (1925), and he has confirmed the observations of previous writers. He also claims to have produced sarcomas in the fowl by implantation of chick-embryo pulp containing high dilutions of arsenic, indol, or tar, and by the intravenous injection of tar in minute quantities prior to the injection of the embryo pulp. These tumours —on a slow-growing osteo-chondro-sarcoma—were transmissible by the inoculation of filtrates, and were similar to the Rous sarcoma in their behaviour both *in vivo* and *in vitro*.

Unfortunately no detailed histological pictures have been given with these important results, and as yet the work does not appear to have been repeated elsewhere. It may be mentioned here that a few experiments with tar on similar lines in this laboratory have not met with success.

Although close contact between the two types of tissue can be obtained, and the two grow side by side in the same tumour, both elements are of necessity cell-grafts. By utilizing the fowl, in which a sarcoma can be produced by injection of a cell-free tumour extract (which tumour may be regarded as spontaneous), there is obviously a closer association between the embryonic implant and the tumour-producing agent.

It should be noted that only that part of the filtrate which was in close association with the embryo fragments was introduced into the host, since particular care was taken to wash the fragments after incubating them with the filtrate.

The resulting tumours showed histologically that the more specialized embryonic tissue, such as cartilage and epithelium, was entirely unchanged by its association with the tumour filtrate. The inference follows that the sarcoma-cells are derived either from cells of the host or from the embryonic connective tissue. This latter possibility could not be entirely excluded on histological evidence, since the embryonic connective tissue merged imperceptibly into, and was indistinguishable from, the surrounding sarcoma.

SUMMARY OF RESULTS.

1. Embryonic structures survived in a rapidly growing Rous sarcoma somewhat longer than in normal muscle. Embryonic elements could be demonstrated in these mixed tumours after 28 days, whereas in all cases where embryo tissues alone were introduced, they had completely disappeared in 14 days.

2. When the embryo tissue tumour was reinoculated into another bird it rapidly disappeared, whereas reinoculation of the mixed Rous and embryo tumours produced a sarcoma in which no embryonic elements could be recognized.

3. Retinal cells treated with "filtrate" produced a spindle-cell sarcoma with small collections of pigment between tumour-cells, some of which contained pigment-granules in their cytoplasm. Reinoculation of the tumour produced a sarcoma in which no pigment could be found.

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THE INFLUENCE OF FERMENT ACTION UPON THE INFECTIVITY OF THE ROUS SARCOMA.

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VARIATIONS in the infectivity of the Rous fowl sarcoma have been noted by several observers. In his original paper Rous describes how the tumour became more virulent and less specific as regards the particular strain of host after passage through a series of Plymouth Rock fowls. In a recent paper Gye and Andrewes (1926) describe variations in the filtrability of the tumour, and record that a tumour which was at first easily filtrable became non-filtrable. Since we started to work with this tumour it has passed through a considerable number of birds. It showed a marked increase in virulence during the first six months or so of passage, but after this appears to have remained on the whole at a maximum virulence, although filtrates prepared from different tumours may show marked differences in their infectivity. Berkefeld filtrates of the tumour have at times given positive results by the injection of 0·001 c.c. contained in 0·5 c.c. of saline (*vide* Exp. 131/26, Fig. 2), while at other times 1 c.c. has failed to produce a tumour.

It appeared to us that variations in the filtrability of the tumour might be dependent upon its physical condition, which varies considerably in different specimens, some being very soft and mucinous and others pale and firmer in consistence. With the idea of altering the physical condition, and possibly liberating the infective agent in a more filtrable form by breaking up protein

particles, the tumour suspension was incubated with trypsin prior to filtration through a Berkefeld candle. This treatment considerably enhanced the infectivity of the filtrate, but not, as subsequent work has shown, by facilitating the passage of the infective agent through the filter.

In the first experiments the tumour was ground with sand and suspended in saline; to this saline suspension was added 2½% of a glycerine extract of the pancreas (Liquor Trypsin Co., Allen & Hanbury). The fluid was then incubated at 37°C. for 2½ hours. The suspension was clarified by filtering rapidly through paper pulp and finally passed through a Berkefeld filter.

The filtrate so obtained was then injected into fowls, and the results compared with those obtained with a filtrate made up in a similar manner, but without the addition of trypsin. It was found that the trypsanized sample appeared considerably more active for tumour production (Table I).

TABLE I.—*Showing Rapid Appearance of Tumours after Inoculation with Tumour Extract Treated with Acid Trypsin.*

Size of tumours indicated by number of + signs. — = no palpable tumours. Results recorded 10 days after inoculation. Birds 142, 143 and 145 subsequently developed tumours.

	Chick No.	
Tumour extract treated with acid trypsin	146	++++
	147	++++
	148	+
	149	+++
	150	++
Untreated extract	141	++++
	142	—
	143	—
	144	—
	145	—

An investigation into the factors concerned in the activation of filtrates of the Rous sarcoma by trypsin has led to a series of observations, which will be detailed in the present and in subsequent papers.

In the first experiment described above no estimation of the H-ion concentration of the fluid was made, but on repeating the procedure and testing the pH of the fluid, this was found to be distinctly acid (about pH 6), due to the acidity of the trypsin solution. Since this degree of acidity is not favourable for tryptic digestion, the question next arose as to whether the activating effect is dependent at all upon a digestive action, or merely upon the presence of some substance (nucleo-protein, glycerine, etc.) contained in the trypsin solution; there was also the further possibility that the alteration in pH alone might determine the effect. On looking into the question of the action of trypsin at various H-ion concentrations, it appeared that although the optimum reaction is pH 8–9, there was a very appreciable action at pH 6; thus, according to a chart given by Michaelis (1914), the action on peptone at pH 6 amounts to about ⅓ of that at pH 8. We tested the action upon casein, and found that a very definite digestion occurs at pH 6.

If the stimulating effects observed were due to tryptic action, it seemed, at first sight, that an adjustment of the reaction to about pH 8 would lead to an increased effect.

The following experiment (Exp. 88/26, Fig. 1) was devised to test this point:

A cloudy filtrate of the tumour was prepared by clarifying a saline suspension of ground tumour as in the experiment mentioned above. This filtrate was

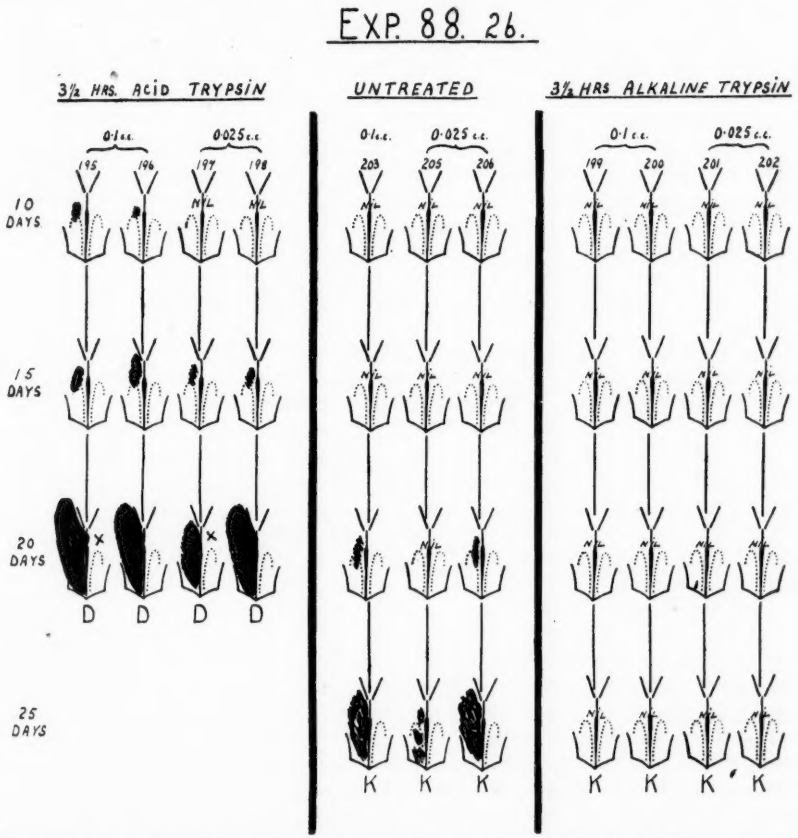

FIG. 1.—*The effect of trypsin upon the infectivity of a Berkefeld filtrate of the Rous sarcoma.*—The first four birds were inoculated with filtrate incubated with an acid solution of trypsin at 37° C. for 3½ hours; the next three birds were inoculated with untreated filtrate; the last four birds were inoculated with alkaline filtrate (pH about 8) incubated with trypsin. The condition of the birds at the end of 10, 15, 20 and 25 days is charted. The last chart of each bird records the *post-mortem* findings and the earlier charts show roughly what was palpable. D = dead by the date indicated; K = killed on the date indicated; X = secondary deposits in internal organs. It is seen that the birds inoculated with acid trypsin developed tumours rapidly and died between the 15th and 20th days, whereas birds inoculated with untreated filtrate survived until they were killed on the 25th day. The filtrate treated with alkaline trypsin produced no tumours.

then divided into three lots. To the first 2½% of the trypsin solution was added, producing a reaction of about pH 6; this was then incubated at 37°C. for 3½ hours. The second sample was untreated, the volume being adjusted by the addition of normal saline, and the fluid kept at room temperature. The third lot was treated with trypsin in the same way as the first, but the reaction was adjusted to about pH 8 with N/10 sodium carbonate.

Each of the above solutions was now passed through a Berkefeld filter, and the clear fluid thus obtained was inoculated into the right pectoral muscles of a series of Plymouth Rock chickens in doses of 0·1 c.c. and 0·025 c.c.

Fig. 1 shows the results obtained; the condition of each bird at the end of 10, 15, 20 and 25 days is charted, the last chart of each bird recording the *post-mortem* findings; previous charts are rough indications of what was palpable during life.

It is seen that the birds inoculated with acid filtrate and trypsin developed tumours rapidly, and all died between the 15th and 20th days; in the birds inoculated with untreated filtrate the tumours developed more slowly, and the fowls all survived in a healthy condition till the 25th day, when they were killed.

The filtrate treated with alkaline trypsin produced no tumours; the birds were killed on the 25th day and the *post-mortem* findings were negative.

It is seen from this experiment that while the acid trypsinization tends to stimulate tumour production, incubation with alkaline trypsin renders the filtrate completely inactive in the doses used. From these results it might be concluded that the reaction of the fluid determined the effect—the addition of trypsin being immaterial. The next experiment, therefore (Exp. 131/26, Fig. 2), was designed to separate the effect of acid trypsin from that of acidity alone, and was carried out thus:

An opalescent filtrate of the tumour, prepared as in the last experiment, was divided into three portions. To the first was added 2½% of trypsin solution, bringing the reaction to about pH 5·5 (= T); to the second dilute acetic acid until the H-ion concentration was brought to the same figure as in the first sample (= A); to the last portion a little normal saline was added (= O). Each of these fluids was warmed to 37°C. for a few minutes and then passed through a Berkefeld filter. Two dozen chickens were now inoculated intramuscularly with the Berkefeld filtrates in the following manner:

The birds were divided into four lots according to the quantities injected, which were 1 c.c., 0·1 c.c., 0·01 c.c. and 0·001 c.c. of the filtrate, the volumes of the last three doses being made up to 0·5 c.c. by the addition of saline. Each bird received two doses, one on either side, and the inoculations were so arranged that different samples of filtrate tested were compared with one another on opposite sides of each bird. This arrangement eliminated any effects due to individual differences in susceptibility amongst the birds, and each sample of filtrate was tested by 16 inoculations—4 for each dose used. Two observations on each bird are recorded: the first after 14 days indicates one's impression of the tumour on handling the birds; the second, charted on the 19th day, when all the fowls were killed, shows the exact extent of the growth found *post mortem*.

The results were rather surprisingly clear cut, and showed the following points:

(1) An acid reaction markedly inhibited the "take" of the filtrate: out of 16 birds inoculated with varying doses of acid filtrate only 2 showed any growth, the remainder being completely negative at the site of this inoculation.

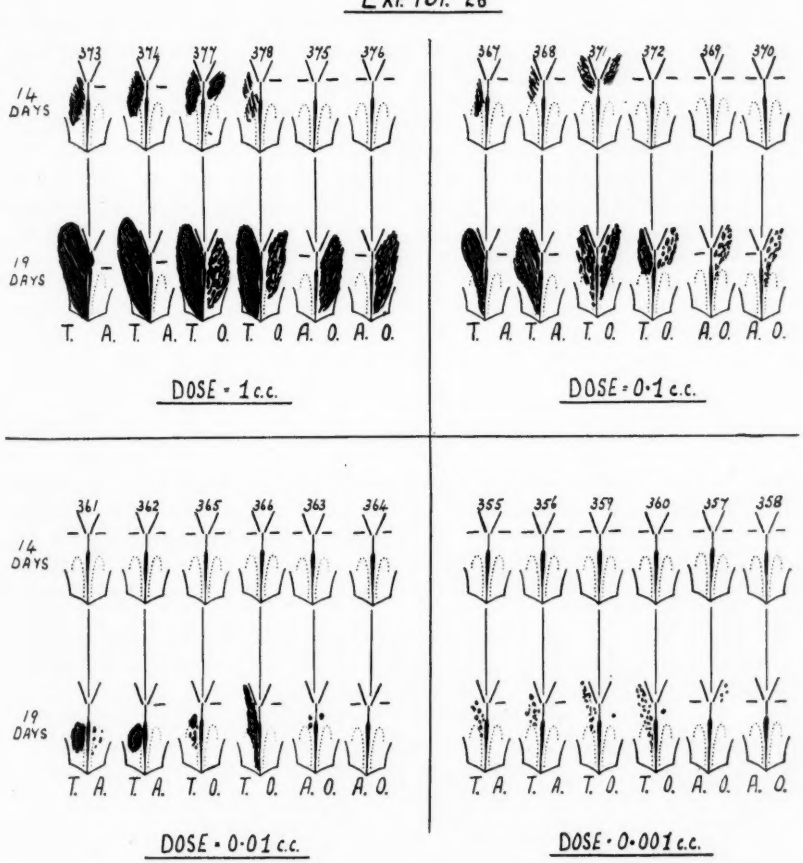

FIG. 2.—*A comparison between the infectivities of filtrates treated with acid trypsin (T), acid without trypsin (A) and untreated filtrate (O).*—Four groups of birds are used corresponding to doses of 1·0 c.c., 0·1 c.c., 0·01 c.c. and 0·001 c.c. of the filtrate. Each bird serves to compare two different solutions as indicated by the letters beneath each diagram. Diagrams charted on the 14th day indicate what is palpable; those charted on the 19th day show exact *post-mortem* findings. It is seen that the acidified filtrate produces only two positives in sixteen inoculations, while the filtrate treated with acid + trypsin has taken in every case, producing more positives and more extensive tumours than the untreated filtrate.

(2) The presence of trypsin in an acid filtrate converts this "acid inhibition" into a stimulation.

Since both the trypsin-treated filtrate (T) and the untreated filtrate (O) produce tumours in the two larger doses, the difference is not so obvious on these birds, although there is a tendency for the tumours on the trypsin side to be distinctly larger; when, however, a comparison is made in the 0·01 c.c. and 0·001 c.c. doses, the difference is more apparent.

Out of 8 inoculations, in these doses, with untreated filtrate only 4 took, 3 of which only produced single foci at the site of the needle puncture; the filtrate treated with acid trypsin, on the other hand, took in every case, producing multiple foci with the smallest dose.

In explanation of the diagrams, it may be as well to call attention to a point in connection with the development of the Rous sarcoma, namely, the variation in appearance with the degree of "take" of the filtrate. When a large dose of a virulent filtrate is inoculated into the pectoral muscles of a fowl, the tumour appears as a diffuse infiltration, often of the whole of the pectoral muscles on the side of the injection. Such diffuse infiltration is indicated in the diagrams by a blacked-in area.

In the case of a non-virulent or an attenuated or diluted filtrate, the growth appears as isolated small foci which finally merge to form a nodular mass, or, if very sparsely scattered, develop into single or multiple rounded tumours. Foci of this sort are indicated in the diagrams by dots.

EFFECT OF H-ION CONCENTRATION UPON THE INFECTIVITY OF THE FILTRATE.

We have tested the reaction of suspensions of fresh Rous sarcoma in normal saline on many occasions, and found it to be about pH 7, or slightly on the acid side of this (pH 6·8). In the above experiment acidification of the filtrate practically destroyed its infectivity. The following experiment (Exp. 155/26, Fig. 3) shows the effects of alterations of H-ion concentration ranging from pH 4·5 to pH 9.

A clear filtrate of the tumour was prepared by filtration through paper pulp and sand; this was divided into a series of samples, and each was adjusted to the required reaction by the addition of a quantity of phosphate buffer. The adjusted samples stood at room temperature for 1½ hours, and were then inoculated into the pectoral muscles of ten fowls. Fluids of different reaction were compared on opposing sides of each bird: each dose contained 0·125 c.c. of the original filtrate. The birds were used in duplicate (*vide* Fig. 3).

All the birds were killed 17 days after inoculation, and the extent of the tumours charted. As in the last experiment the result was quite clear cut, and showed the following points:

(1) No tumours were produced when the reaction was more acid than pH 6, and there was a definite decrease in infectivity at pH 6.

(2) There was an increase in the infectivity as the alkaline end was approached, this being indicated by more abundant and closely packed tumour foci.

DISCUSSION.

From the above experiments it is seen that the infectivity of filtrates of the Rous sarcoma may be modified by alterations in H-ion concentration and by the addition of trypsin. Although in the original experiments the treatment was carried out upon the tumour emulsion prior to filtration, subsequent work has shown that the alterations in infectivity could be as easily produced by treatment of the fluid *after* filtration. As is seen in Fig. 3, the alterations in pH were made subsequent to filtration; other experiments, to be detailed in a later communication, have shown that trypsin will produce its effects when added to the completed filtrate. The facts which emerge from the above experiments may therefore be summarized as follows:

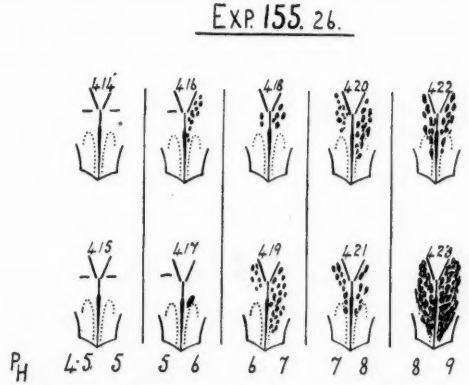

FIG. 3.—*Showing the effect of H-ion concentration upon the infectivity of the filtrate.*—The experiment is duplicated, the upper and lower rows being treated identically. Samples of filtrate were adjusted to the required reaction, the pH of which is indicated by the figure below on each site of inoculation. All the birds were killed on the 17th day. The tumours are in an early stage and appear as multiple foci. The intensity of the "take" can be judged from the number and size of the foci. It is seen that the two most acid samples (pH 4·5 and 5) fail to produce tumours, and that the sample at pH 6 produces a poor "take" in comparison with that at pH 7. There is a definite improvement in the "take" as the alkaline end is approached.

(1) A comparatively slight change in the reaction of the filtrate towards the acid side (pH 6) inhibited the production of tumours; on the other hand an alteration towards the alkaline side (pH 8–9) gave a considerable increase in the infectivity.

(2) The addition of a solution of trypsin reverses these effects, so that the acid trypsin stimulates while the alkaline trypsin markedly inhibits tumour production.

It is well known from the work of Rous and others that the injection of Kieselguhr or other foreign substance along with the filtrate markedly encourages tumour production; such bodies appear to act by stimulating cell

proliferation. In the above experiments the problem appears to be of a different nature, and it seems necessary to assume a more complex mechanism in explanation of these somewhat anomalous effects. Alterations in H-ion concentration might act in various ways: it is very probable that a marked acidity (pH 4 or less) would destroy a living infecting agent, but in the case of a slight acidity (pH 6) such an action is less probable. The fact that the addition of trypsin to a filtrate at pH 6 reverses the "acid inhibition" practically eliminates the possibility that such an inhibition is due to a lethal effect of this sort.

As regards the action of trypsin, it is well known that the processes of autolysis rapidly destroy the infectivity of tumour-tissue and of cell-free filtrates, so that it is not surprising that digestion with alkaline trypsin should act in the same way. It is more difficult to find an explanation for the stimulating action of acid trypsin. This effect might be due, not to ferment action, but to the activating effect of some substance (nucleo-protein, glycerine, etc.) contained in the trypsin solution. Against this explanation are the facts that—

(1) Various preparations of trypsin (fluid and powder) have been used, and all have the same action.

(2) If the ferment action is destroyed by heat the effect is no longer obtained.

THEORY OF THE *RÔLE* OF FERMENT ACTION.

Taking these and other points into consideration, we have been driven to the following conception of the *rôle* played by ferments in the production of the Rous tumour by the cell-free filtrates. The filtrate is normally subjected to an autolytic or digestive process, which may start *in vitro*, but, as is reasonable to suppose, and as we shall produce experimental evidence for later, continues in the fowl after inoculation.

This digestion is essential to the "take" of the filtrate, and factors such as alteration of the pH to the acid side which inhibit or modify tryptic action lessen the filtrate's infectivity; on the other hand, a too rapid digestion either *in vitro* or *in vivo* is equally fatal to the production of a tumour.

The above may be stated in other words as follows:

The conditions for tumour production by the filtrate depend upon a nicely balanced autolytic process. Factors which disturb this process will alter the infectivity of the filtrate.

If the above conception be true, it is clear that the factors which modify ferment action will influence the infectivity. These factors are:

(*a*) Ferment content.

(*b*) H-ion concentration.

(*c*) Temperature.

(*d*) Time for which filtrate is treated before it is inoculated into the bird.

These are to be regarded as the four variables, and an alteration in one may be counterbalanced by an opposing change in another. This furnishes us with an explanation of the reversal of effect when trypsin is added to an acid filtrate. Here the inhibitory effect of the acid reaction is counterbalanced by the addition of an excess of ferment; on the other hand, the alkali reaction already carries the autolytic digestion far enough and the addition of more ferment is fatal.

This interdependence of factors must be borne in mind; for instance, unless the conditions of pH, temperature and time are defined, it is impossible to gauge the effect of altering the ferment content. In a similar way the effect of an alteration of pH depends upon the other factors; *e.g.* in the experiment in Fig. 3, the samples of filtrate are maintained at the various pH values for 1½ hours at room temperature before injection; when the conditions as regard time and temperature are altered, different results may be obtained, as will be shown in a later communication. Even with the conditions simplified as much as possible, however, it is often difficult to control adequately all the factors; for instance, the enzyme content is usually not known except in solutions to which a relatively large quantity of ferment has been added.

The interdependence of these factors appears to explain the results of E. Harde (1926).

This worker in a short communication describes an experiment in which a filtrate of the Rous sarcoma was made acid (pH 5·5) by the addition of potassium acid-phosphate and compared with an alkaline filtrate (pH 7·5–7·8). It was found that the acid filtrate produced a larger and more rapidly growing tumour than the alkaline filtrate.

This result appears to be exactly opposite to our findings in the above experiments, but since in Harde's experiment the conditions as to temperature and time are not stated, it is impossible to draw a comparison between the two observations.

It appears to us that the discrepancies between the results of different workers upon the Rous sarcoma, and particularly in relation to Gye's work, can be accounted for by a lack of control of the factors mentioned above, especially by a failure to control the H-ion concentration.

As regards the interpretation of experiments in which chloroformed or otherwise inactivated filtrates of the Rous sarcoma are rendered active by the addition of tumour cultures, it seems possible that an alteration in ferment content or in the conditions controlling ferment action would furnish an explanation.

CONCLUSIONS.

The infectivity of filtrates of the Rous sarcoma is influenced by the factors which control ferment action, *i.e.* H-ion concentration, ferment content, temperature and time: variations in any one of these factors may either greatly increase or entirely prevent tumour production.

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CORRIGENDUM.

In Dr. Axel Höjer's paper (1926, **7**, 356) on a "Method for Determining the Anti-scorbutic Value of a Foodstuff," etc., the following corrections should be made:

Page 358, *line* 13, delete "together with a titrated series for comparison."

Page 358, 3 *lines from bottom*, delete "or 18 to 36 pints in the case of children."

Page 359, *line* 3, delete "or 4 to 7 pints for children."